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THE BEHAVIOUR OF MINNOWS (*PHOXINUS PHOXINUS* L.) IN A LIGHT GRADIENT

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(*Received* 16 *September* 1955)

INTRODUCTION

It is well established that light can be a factor influencing the behaviour and the distribution of fish. Three centuries ago Izaak Walton wrote: 'For you are to note, that the great old Trout is both subtil and fearful, and lies close all day, and does not usually stir out of his hold', and 'In the night, the best Trouts come out of their holes... draw your bait over the top of the water to and fro' and if there be a good Trout in the hole, he will take it, especially if the night be dark,—for then he is bold, and lies near the top of the water'. In recent years there has accumulated a considerable number of field observations of the diurnal movements of fish suggesting correlations with changes in light intensity. The modern development of echo-sounding techniques for fish detection has demonstrated considerable vertical migrations of fish from upper waters at night into deeper waters during the day. Such vertical migrations occur both in the sea (Krefft & Schüler, 1951) and in fresh waters (Schärfe, 1951) and have been related to definite light intensities (measured in arbitrary units) for certain marine pelagic fish (Richardson, 1952). A horizontal migration into shallow, inshore feeding grounds as light intensities decreased at sunset has been described for yellow perch, *Perca flavescens* Mitchill. (Hasler & Bardach, 1949; Scott, 1955), and for rock bass, *Ambloplites rupestris* Raf. and suckers, *Catastomus commersonii* Lac. (Spoor & Schloemer, 1938) in North American lakes; with increasing light intensities after sunrise, the fish moved offshore again into deeper waters.

Relatively little work has been carried out analysing the causal relationships between such movements and changes in illumination. The present experiments, which form part of a laboratory study of the behaviour of fish in relation to various light stimuli, were carried out at the suggestion of Dr F. R. Harden Jones, who very kindly allowed me to use a light gradient tank which he had constructed.

MATERIAL AND METHODS

The fish used in the experiments were adult minnows, *Phoxinus phoxinus* L., from 5·0 to 6·6 cm. long, caught in the River Cam. They were kept in glass accumulator jars filled with water which was constantly aerated and changed at regular intervals. The fish were fed daily on *Tubifex* and, under these conditions, remained in a healthy condition throughout the experiments.

All experiments were carried out in a blacked-out room at the Zoological Laboratory, Cambridge. The apparatus used consisted of a Perspex trough 360 cm. long and 15 cm. wide, filled with water to a depth of 7·5 cm. Sheets of frosted glass were laid end to end on a frame 15 cm. above the water surface, and over these were placed sheets of thin black cardboard which were bent in such a way as to form a light-proof tunnel extending the length of the trough. An electric light, enclosed in a metal box, was placed at each end of the tunnel and mounted so that the trough was illuminated by diffuse light passing down through the frosted glass. The lights at either end of the tank could be switched on separately or both together. An attempt was made to eliminate some of the directional properties from the gradient by including vertical black paper screens between the frosted glass and the water-level inside the tank. The screens, being continued to bench level outside the tank also served to subdivide the tank into twenty units, or compartments, for purposes of observation.

The intensity of the light falling vertically on to the water surface was measured at a number of points in the tank with a photomultiplier described by Jones (1956), which was sensitive to 10^{-3} metre-candles (m.c.). The distribution of light intensity in the gradient tank is shown in Fig. 1.

Observations showed that for a period varying from 4 to 12 hr. after being placed in the gradient tank the minnows swam up and down the trough at abnormally high speeds, often turning rapidly. After this time the fish appeared to become adapted to the tank, swimming more steadily and in a less confused manner.

The experimental procedure adopted was to place a single fish in the gradient tank one evening and leave it overnight with the light shining at one end of the tank. On the following morning the observer entered the dark room as quietly as possible and sat in a position about 3 ft. away from the centre of the tank. After the observer had been in the room for about 15 min., the successive positions of the fish in the tank at 10 sec. intervals were recorded in pencil on a small pad of paper, the observer remaining quiet, and moving as little as possible in order not to disturb the fish. The 10 sec. time intervals were determined by use of a Palmer time-clock connected to muffled earphones which produced a just audible click; this timer was left running throughout the time that the fish was in the experimental tank and had no apparent effect on the behaviour of the fish.

During each experimental period the position of the fish in the tank was recorded for up to an hour, the lights were then reversed, so that the light now shone at the previously dark end of the gradient, and the fish was left for 2 hr. before a further period of observations was made.

In all experiments, except those with hungry fish, a small number of *Tubifex* were present in the tank. These were placed in all parts of the tank in order that results might not be influenced by the distribution of the worms. Well-fed fish generally appeared to ignore the *Tubifex*, and only on a few occasions were such fish seen to snap up a worm. No water circulation was run during the day when experiments were in progress, but the water in the tank was completely renewed each evening. Using this procedure it was possible to obtain uniform and repeatable results. The

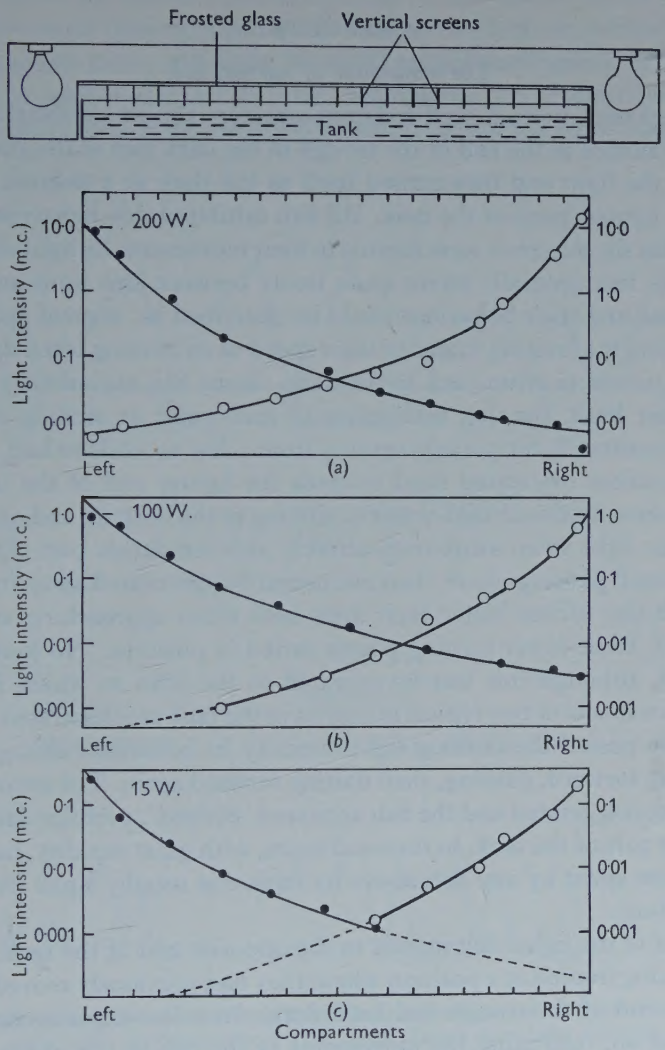

Fig. 1. Diagram of the light gradient tank and the distribution of the vertical light intensity in the tank using different light sources: (*a*) 200 W., (*b*) 100 W., (*c*) 15 W. In each graph the distribution for illumination from the left-hand end of the tank is shown as – • – and from the right-hand end as – o –.

temperatures at each end and in the middle of the tank were usually taken after each period of observation. On some occasions slight temperature differences of about 1° C. were observed, but the behaviour of the fish did not show any consistent relationship to such temperature gradients.

The results presented below are based upon 208 experiments involving between 60,000 and 70,000 recordings.

RESULTS

The behaviour of normal fish

In the gradient tank, normal, well-fed minnows were almost constantly active. Fish most usually turned at the end of the trough in the dark part of the gradient, but on approaching the light end they turned back to the dark at a definite point before reaching the lightest parts of the tank. All fish exhibited this behaviour, and it was considered that the minnows were limited in their movements by light above a certain intensity. The fish generally swam quite freely between this limit and the darker end of the tank and their behaviour could be described as 'normal and unexcited'. On approaching the limiting intensity their speed of swimming often decreased, and they usually turned to swim back to the dark. Some fish moved very slowly when almost at their limit, pausing motionless in mid-water or sinking slowly to the bottom, to remain still, for periods varying from a few seconds to half an hour. All fish in the gradient orientated head towards the lighter end of the tank (positive phototaxis) when inactive in mid-water or sinking to the bottom, and only orientated away from the light when swimming actively into the darker part of the tank. No fish was observed pausing, more than momentarily, orientated away from the light. Although fish also turned below their limit both when approaching and retreating from the light, these lower turning points varied in position. No lower light limit was observed, although this was investigated to the limit to which fish could be seen. The movements of two typical minnows in the tank are illustrated in Fig. 2*a*, *b*.

When a fish passed the limiting light intensity its behaviour changed. It swam jerkily, darting forward, pausing, then darting forward again. The swimming movements seemed exaggerated and the fish appeared 'excited', perhaps darting forward to the lightest part of the tank, to turn and swim, with great rapidity, back below the limit. The time spent by any fish above its limit was usually small (from 0 to 5 % of the total time).

On reversal of the lights fish moved to the opposite end of the tank, their movements now being limited at a position where they had previously moved freely when the newly lit end of the trough had been dark. In a few experiments both lights were switched on, restricting the movements of the fish to the centre of the tank. Immediately after the reversal of lights fish exhibited a 'shock-reaction' initially going into the darker parts of the tank then later venturing farther and farther into the light. During this period the fish appeared rather excited, moving rapidly, and turning often, but after about 15 min. they had usually begun to assume their normal behaviour and were swimming freely below their limit. Selection of the limit varied with individuals but often tended to improve with time, as is shown in Fig. 2*c*.

The minnows appeared to maintain their position at or below their limit by a comparison of light intensities. From time to time a fish has been observed to have a well-established limit at one point in the tank and to stay motionless in mid-water at this point. Respiratory movements (the 'jetting' of water from the opercula)

would slowly move the fish to a position perhaps 6 cm. farther up the tank into the light. The fish would then turn and swim back to the dark, or, occasionally, maintaining its position facing the light, it would propel itself gently backwards with its pectoral fins until it reached its limit, whereupon the reversed pectoral beats ceased. This latter reaction was totally different to the normal swimming movements

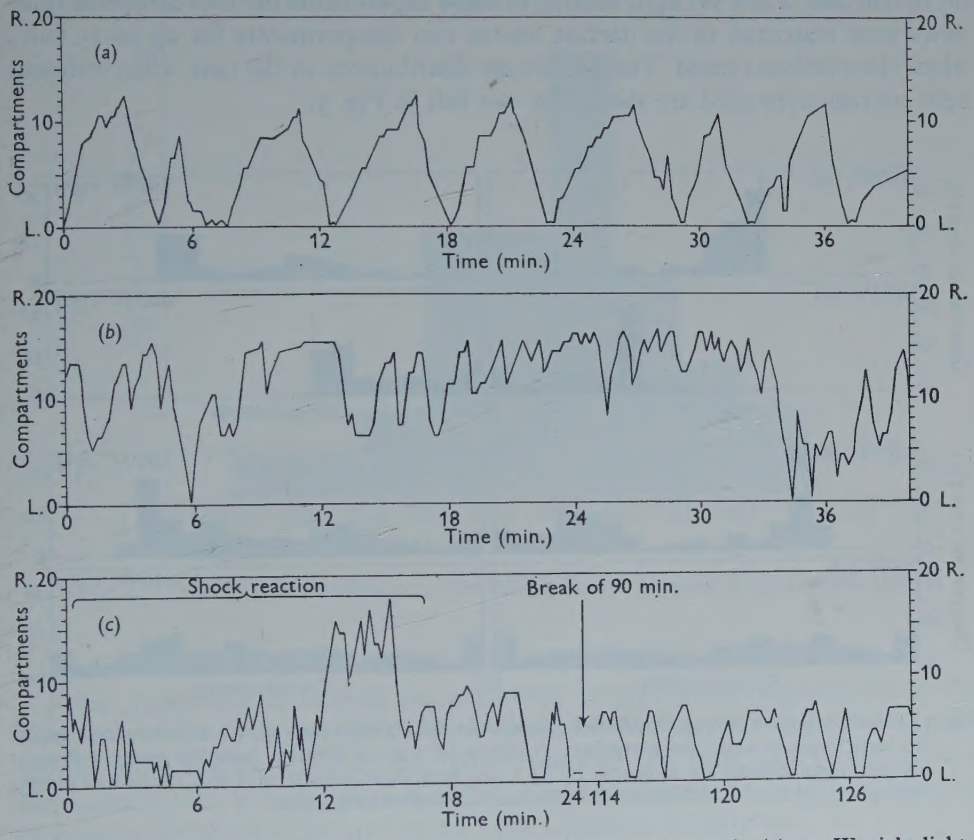

Fig. 2. The tracks to and fro of three typical well-fed minnows in the tank: (*a*) 100 W. right light; (*b*) 100 W. right light, (*c*) the tracks of a fish immediately after light reversal, showing 'shock reaction', followed by the movements of the fish 90 min. later.

of minnows seen in this apparatus, and was a most convincing demonstration of the presence of a light limit. Such behaviour must depend upon an actual comparison of intensities by the fish.

As the light intensity in the gradient decreased roughly in inverse proportion to the square of the distance from the source, it was possible that the minnows might have been responding to a given rate of change of light intensity rather than to any particular intensity. This possibility was investigated by varying the strength of the source, using different electric light bulbs in different experiments. In this way the position of any intensity could be moved up or down the gradient, although the rate of change of light intensity remained the same (Fig. 1). The results of these experiments

showed that the fish were responding to light intensity alone; as the limiting intensity was moved along the tank, the length of tank in which the fish moved freely and unexcitedly increased or decreased accordingly. In certain experiments the limiting effect of light in this apparatus was brought out particularly clearly when individual fish were 'held' in the darkest part of the tank by using a 500 W., or, in one case, a 200 W. light source; in these experiments the movements of minnows were restricted to the darkest one or two compartments for up to 45 min., when observations ceased. The percentage distributions in the tank when different light sources were used are shown for two fish in Fig. 3.

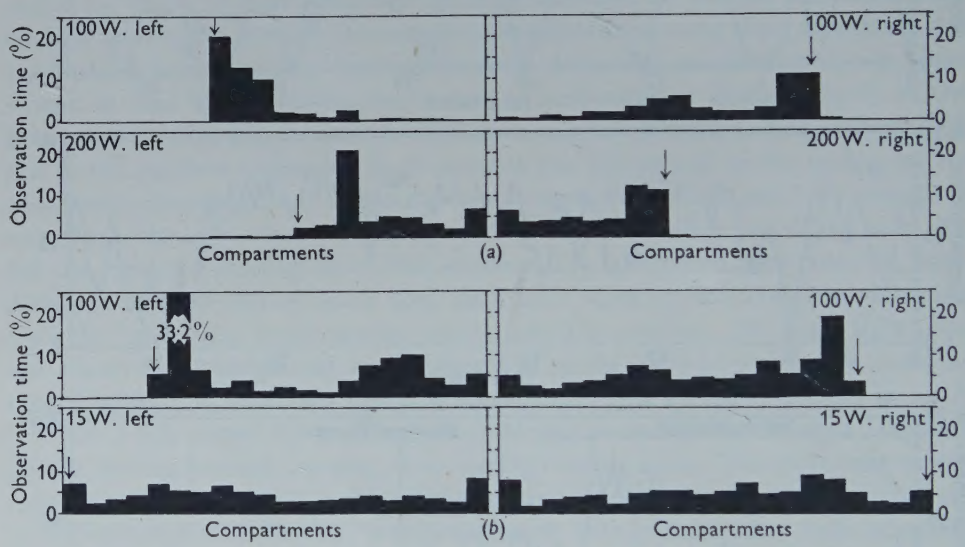

Fig. 3. The distribution of typical well-fed minnows in the gradient tank shown as the percentage of the time spent in each compartment. Positions of a given limiting intensity using different strength light sources are indicated by (↓): (*a*) four distributions of a fish limited at about 0·030 m.c.; (*b*) four distributions of a fish limited at about 0·14 m.c.

Each minnow had its own limiting intensity, which might differ from those of other fish. A different limit was also demonstrated for the same fish on different occasions; however, individual fish held the same limit for at least 48 hr. during experiments. The distribution of the values of limiting light intensities observed for normal, well-fed minnows is shown in Fig. 4. Although these values are mainly derived from different fish, some values of different limits observed in the same individual on different occasions have also been included. Each limit included in this diagram has been derived from the results of from two to six experiments, in which the same limit was maintained by an individual fish.

During the course of earlier experiments all fish observed in the gradient tank were kept in the dark room for some days or even weeks before being used in experiments, in order to eliminate the vestiges of possible diurnal activity rhythms, which it was thought might occur. Later experiments were carried out with fish taken

directly from aquaria which had been exposed to normal diurnal changes in illumination. No difference was observed in the behaviour of these fish, the light limits in the gradient being the same for both types of fish.

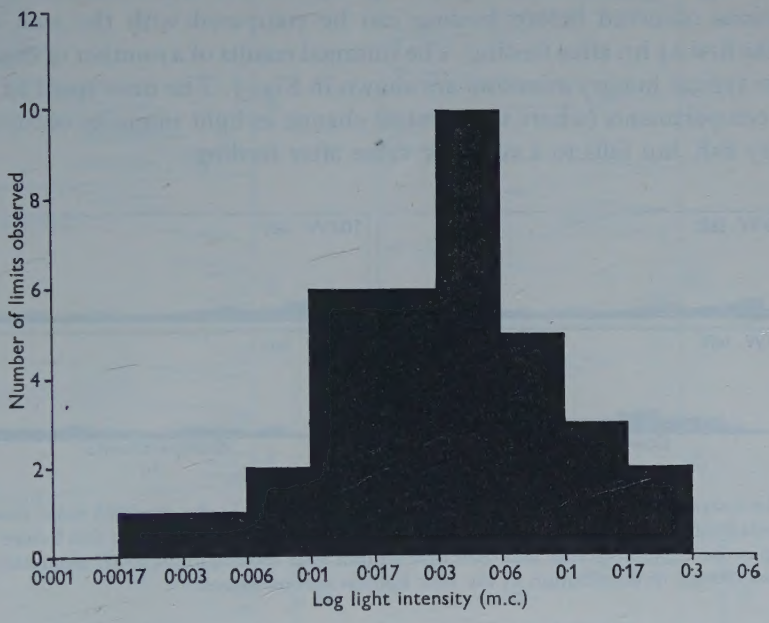

Fig. 4. The frequency distribution of the light limits of minnows in the gradient tank.

The effect of hunger on behaviour

Jones (unpublished) carried out a number of preliminary experiments on the behaviour of fish in the gradient. From his results he suggested that whereas well-fed fish were mainly found in the darker parts of the tank, hungry fish spent a considerable time at high light intensities. Further experiments were performed to investigate the effect of hunger on the light responses of minnows.

The fish used in this series of observations had not been fed for about 10 days preceding the experiments. The fish were observed by the standard procedure, except that no *Tubifex* were present in the tank. All experiments with hungry fish were carried out using 100 W. lamps as light sources. After the behaviour of a hungry fish had been observed for at least one day, a small ball of *Tubifex* was placed in a lighter part of the tank. The food was left in the tank for about 20 min., although the fish ate most of the worms in a very short time; after this period any worms remaining were siphoned out of the tank. Observations were resumed 2 hr. later.

Jones's results were fully confirmed. Hungry fish spent much of their time in the highest light intensities in the tank, making repeated excursions into that region. Indeed, on two occasions a hungry fish remained in the lightest compartment of the tank for up to half an hour (when observations ceased); such behaviour was never observed at any time during the course of the experiments with fed fish. After

feeding the minnows retired to the darker parts of the tank for about 24 hr., after which they showed an increasing tendency to return to the light with time.

Although the lights were regularly reversed during these experiments, by referring the percentage distribution of the fish to the lighted end of the tank, the sum of the distributions observed before feeding can be compared with the sum observed during the first 24 hr. after feeding. The summed results of a number of experiments with two typical hungry minnows are shown in Fig. 5. The time spent in the four lightest compartments (where the greatest change in light intensity occurs) is high in hungry fish, but falls to a very low value after feeding.

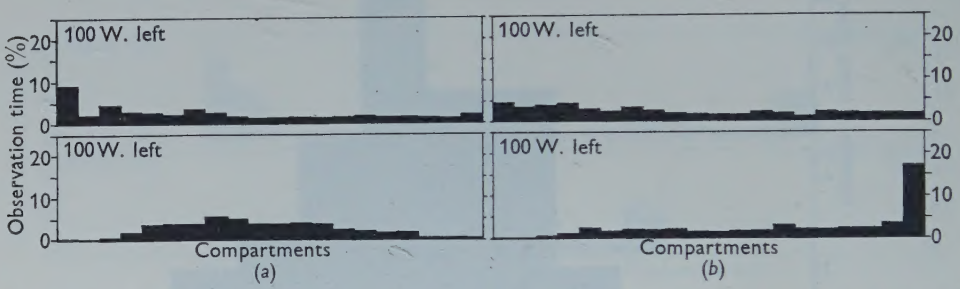

Fig. 5. The distributions of two typical hungry fish (*a*) and (*b*), in the gradient tank, shown as the percentage of the time spent in each compartment. The distribution of the fish before feeding is shown in the upper diagrams, and their distribution after feeding in the lower diagrams. Note the marked change in distribution in the four lightest compartments.

The behaviour of minnows prior to spawning

In mid-April, immediately prior to their breeding season, shoals of minnows were seen on sunny days swimming at very high light intensities in shallow streams around Cambridge. These observations were in contrast to the experimental results obtained with minnows in the light gradient, and it was decided to carry out further experiments with prespawning fish in this apparatus. The fish used in these experiments had been kept in aquaria in the Cambridge Zoological Laboratory for at least 7 months prior to the experiments, and belonged to the same stock from which fish were taken for earlier work. These fish had begun to assume nuptial coloration, particularly noticeable being the milk-white spots at the bases of the paired fins and on the opercula, described by Frost (1943) as characteristic of the spawning livery.

Unlike the minnows examined in the late autumn and winter, these prespawning fish swam rapidly up and down, turning often and entering all regions of the gradient. Although they generally showed a tendency to spend a greater time in the darker parts of the tank, their excursions into the lightest parts were frequent and little evidence of any fixed light limit was shown even when strong light sources were used. These fish examined in April were much more active than the previous fish examined in the winter, as can be seen by comparing the movements of the two typical prespawning fish shown in Fig. 6 with those of the fish shown in Fig. 2.

The method of recording the movements of minnows in the gradient tank, and later plotting these results against time, allows calculation of the speeds of swimming

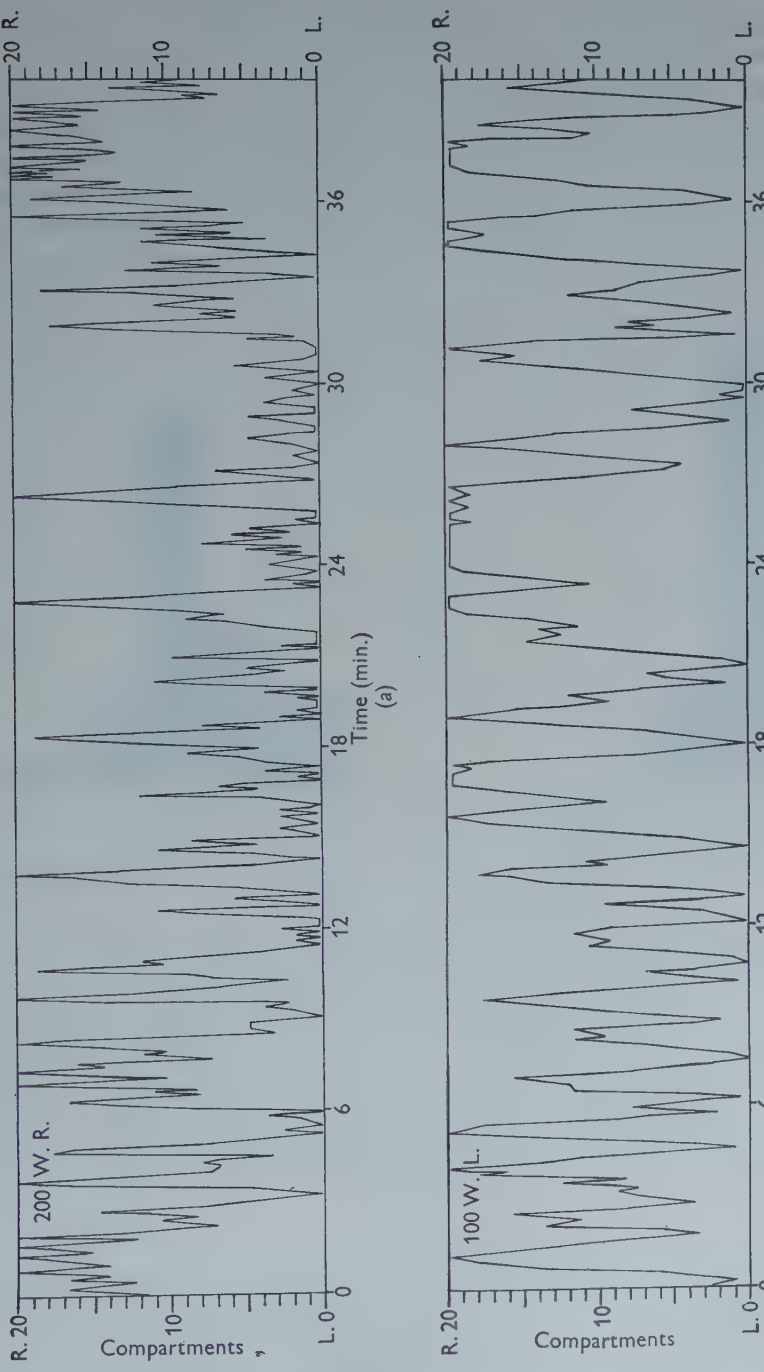

Fig. 6. The tracks to and fro of two typical minnows in the gradient tank observed in mid-April, prior to spawning: (*a*) 200 W. right, (*b*) 100 W. left.

of fish in the apparatus. In this way the activity of these minnows just prior to spawning, as expressed by mean swimming speeds in single experiments, may be compared with the activity of minnows in December. The mean swimming speeds observed in twenty-four experiments on prespawning minnows made between 11 and 22 April 1954 have been compared with similar results for 24 hr. experiments made in December 1953. The December experiments were taken 'as they came', but the two series are strictly comparable since equal numbers of experiments using the same light sources and illuminated from the same end of the tank have been used. The mean swimming speed for the December fish was 2·57 cm./sec.,

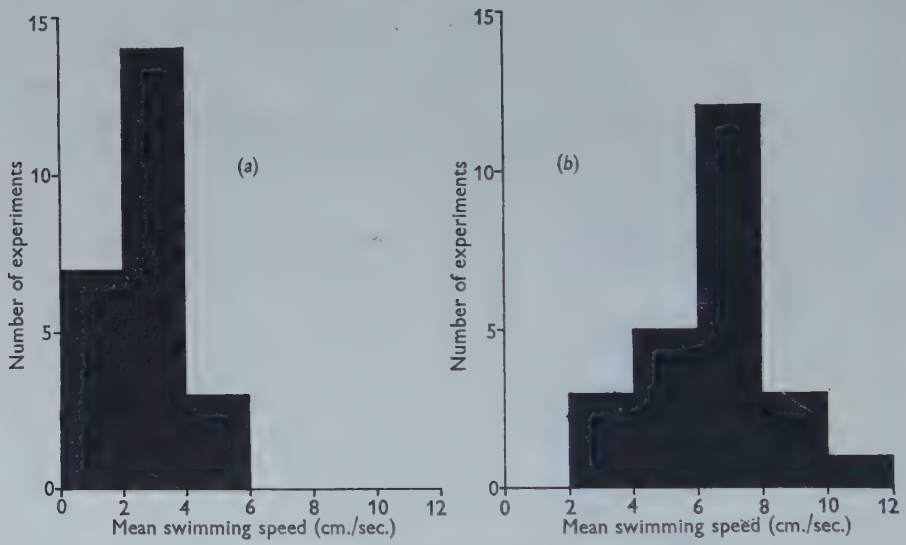

Fig. 7. The frequency distribution of the mean swimming speeds of fish observed in December (*a*) and in April (*b*) in the gradient tank.

whereas the mean swimming speed for the fish examined in mid-April was 6·75 cm./sec., an increase of 159%. The distribution of these results for the December and April series are shown in Fig. 7. The temperature of the water was 17–19° C. during the December experiments and 18–20° C. during the mid-April experiments, and it seems improbable that the difference in activity observed at these different times of year can be attributed to the small difference in temperature.

No attempt was made to distinguish male from female fish in these experiments and possible differences in the activity of the two sexes have been ignored.

DISCUSSION

At the time of completion of these experiments Trincker (1954) published diagrams of the movements of a shoal of goldfish in an apparatus which would appear to act as a type of light gradient. Although his work was carried out for different purposes, the movements of the shoal in the control experiments strongly resemble the movements of minnows in the light gradient. Like single minnows, the goldfish

shoal was constantly active but did not swim into the lightest parts of the apparatus, apparently being limited in their movements by light above a certain intensity. Trincker also conditioned the goldfish to feed in the lightest parts of the tank. The conditioned fish then modified their normal behaviour pattern, spending several hours at high light intensities. Hungry minnows in the gradient similarly failed to respond to light intensities which they avoided when well-fed. It is not possible to determine from the present experiments whether this reaction was innate, or due to a previously learnt association between light and food.

Hoar, MacKinnon & Redlich (1952) observed the behaviour of small groups of Pacific salmon fry and smolt in a light gradient tank. Their results were presented simply as the percentage distribution of the fish in the lighter and darker halves of the tank. It is apparent that different species, and different stages within the same species of salmon, differed in the percentage of the time spent in the lightest half of the gradient, but the method of presentation does not indicate whether the salmon, like the minnows, had a definite light limit. Similar light gradient experiments were carried out by Kawamoto & Nagata (1952), although in their apparatus the light was not diffuse. No evidence of a limit was shown for either of the species studied, but, in view of the observations on the initial behaviour of minnows after being placed in the gradient, it seems probable that the results of Kawamoto & Nagata are based largely on the observation of shock reactions in the fish.

Schärfe (1953) has reviewed a number of field experiments on the reactions of fish to electric lights. Fish, singly, or in shoals, moved away from a light shone on them; there was often an initial shock-reaction, but a final position was taken up at a definite distance from the light; this distance varied with species and with physiological state within a species. Varying the intensity of the light proportionately increased or decreased the distance of the fish from the source. The results of these experiments suggest that the fish were limited by light above a certain intensity but, also, that they had a positive phototaxis orientating their movements towards the light source. In all these field experiments the lights used would act as point sources; nevertheless, the results seem to agree well with the results of the light gradient experiments where diffuse, although directional, illumination was used.

In experiments with a number of minnows in a 'split-tank', half of which had been covered to exclude the light, Jones (1956) found that the fish remained in the darkened half of the tank until the light intensity outside was reduced to between 0·17 and 0·08 m.c. Below this intensity minnows crossed freely into either half of the tank. It seems that the minnows in the split-tank were limited in their movements by light above about 0·1 m.c., a value which agrees well with that observed in the light gradient.

Discrimination of the light limit in the gradient tank was very fine. A change of one-tenth of a log unit at 0·002 m.c. or 0·006 m.c. was readily and repeatedly detected by individual minnows. These results are perhaps surprising, in view of the careful work of Bull (1928, 1932) with marine fish. In *Labrus bergylta*, Bull (1928) found that discrimination could only be made with difficulty between 7500 and 80 m.c.; in later experiments (Bull, 1932) with *Blennius pholis* he found that the fish could not

be conditioned to discriminate between 20,000 m.c. and intensities down to 36 m.c., although discrimination was made between higher light and 20 m.c. He concluded that intensity discrimination in fish was extremely poor.

The apparent discrepancy between the present results and those of Bull probably lies in the lower order of the light intensities used in the gradient tank. The visual range of fish extends well below the light limit described here, and, in view of Bull's work, it is reasonable to conclude that the limit to the movements of the fish was also the limit (threshold) to some physiological mechanism, rather than the response to one intensity selected from the middle of a range which could all be perceived.

Scharrer (1928) conditioned blind minnows to respond to light. The fish only showed a response when the head was illuminated, and removal of the pineal and associated parts of the mid-brain, the pineal-complex, abolished the response. Scharrer obtained consistent reactions from fish at intensities down to 0·5 m.c., whilst the lowest intensities at which he observed responses in particularly well conditioned individuals were 0·0226 and 0·017 m.c. If these results are compared with Fig. 3, showing the distribution of limits in the gradient, it is apparent that the threshold for pineal stimulation and the limiting light intensity are of the same order. These results suggest that the pineal might be the receptor controlling the limit of minnows in the gradient.

Certain evidence derived from experiments on other fish lends support to this suggestion. Hoar (1955) has investigated the role of the pineal organ in the phototactic responses of the sockeye salmon smolt *Onchorhynchus nerka*. Unoperated smolt were described as 'negatively phototactic'; this was expressed as the percentage of fish found in the exposed half of a circular tank, half of which had been artificially darkened. Blind smolt had essentially the same percentage distribution as the controls, whereas the distribution of blind fish in which the pineal had also been destroyed approached random. In similar 'split-tank' experiments Breder & Rasquin (1947) related the sign of the 'phototaxis' to the degree of exposure of the pineal region in the blind Mexican characins belonging to the genus *Anoptichthys*. Later (Breder & Rasquin, 1950) they extended the relationship to a number of normal, eyed teleosts. Kuhn & Kähling (1954) have made a more detailed study on one of the blind characins, *Anoptichthys jordani* Hubbs & Innes. They conditioned the blind fish to respond to a light stimulus and found that both young and older fish had the same threshold for stimulation, despite marked differences in the state of degeneration of the optic cysts. Whilst they point out that the sensory receptors for this reaction are still not known, it is of interest that the threshold for stimulation is about 0·12 m.c., a value similar to that found by Scharrer (1928) for stimulation of the pineal in blind minnows.

The possibility that the light limit in the gradient tank is the threshold for the change from rod to cone vision in the eye, rather than the pineal threshold, cannot be ignored. From the results of experiments on visual acuity in the minnow, supported by observations on retinal histology, Brunner (1935) concluded that the change-over from rod to cone vision occurred between 0·008 and 0·002 m.c. This value is lower than the majority of the limits observed in the gradient, and a log unit

lower than the mean of these limits. However, in the sunfish, *Lepomis*, Wolf & Zerrahn-Wolf (1935) found the threshold for cone vision, as determined by flicker fusion studies, to be 0·4 m.c.; a value as much greater than the gradient limits as Brunner's result was below them. Clearly, further work is required to determine the true nature of the receptor for the light limit in the gradient, but so far it has not been possible to carry out the critical experiments with blind fish.

The increased activity prior to spawning observed in minnows in the light gradient, agrees well with field observations. The experiments were made about 3 weeks before the peak spawning dates reported by Frost (1943) for minnows in Windermere. Frost observed considerable activity in the fish at this time; they migrate from lakes to running waters, from deeper rivers into shallow side streams, and may leap clear of the water in attempting to pass small barriers.

The experiments described here were mainly carried out in the late autumn and the winter. Frost (1943) reports that in Lake Windermere minnows are not seen in open water at this time, but are found under stones. However, in the summer, she found shoals of non-breeding minnows swimming in surface waters only a few feet deep. Unfortunately, it was necessary to terminate the gradient experiments in the early spring, but it may be well to note the possibility that a fit between the behaviour of individual fish under the very specialized conditions in the gradient tank and the observed behaviour of minnows in the field, might not have been obtained had experiments been carried out in the summer months.

SUMMARY

1. Minnows in a light gradient tank were almost constantly active, but were restricted in their movements by light above a certain intensity.

2. The value of the limiting intensity differed for individual fish but lay between 0·2 and 0·002 m.c. These values are of the same order as the threshold for light stimulation of the pineal complex in blind minnows, described by Scharrer. It is suggested that the pineal might be the receptor for the light limit, and evidence supporting this suggestion is discussed.

3. Hungry minnows were not limited by light, and spent a considerable percentage of the observation time in the lightest parts of the tank. After feeding they assumed normal limited behaviour.

4. In mid-April, prior to spawning, minnows examined in the gradient were extremely active and showed little sign of a light limit. The mean swimming speed of fish in mid-April was 159 % faster than that of fish observed in December.

I wish to express my thanks to Prof. Sir James Gray for allowing me the privilege of working in his laboratory at Cambridge. My grateful thanks are due to Dr F. R. Harden Jones for allowing me to use the light gradient, and for the use of his photomultiplier, and especially for his constant advice and encouragement throughout this work. This work was carried out during the tenure of a Development Commission Fisheries Training Grant, which is gratefully acknowledged.

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THE BEHAVIOUR OF MINNOWS IN RELATION TO LIGHT INTENSITY

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INTRODUCTION

Many freshwater and marine animals appear to have an optimum range of light intensity. Under natural conditions pelagic species keep within their range by making a vertical migration from deep to shallow water during the evening and returning to deep water the next day. Reversed migrations are also known. While the pattern of migration can be correlated with changes of light intensity, the reactions of individuals cannot be studied easily in the field, and there is the question as to how the animals find their optimum and remain within it as it moves up and down the water column. Cushing (1951) has reviewed the problem for the planktonic crustacea, but so far little work has been done with fish. Observations were therefore made on the locomotory activity, shoaling and feeding behaviour of minnows in relation to light intensity to add to an understanding of the responses of fish to light.

MATERIAL

The minnows, *Phoxinus phoxinus* (Linn.), used in the experiments were caught in the River Cam and kept in a stock aquarium exposed to normal variations in light intensity. They were fed on chopped liver, living *Tubifex* and *Daphnia*. As judged from their lengths (Frost, 1943), they were in their second or third years and were sexually mature.

LOCOMOTORY ACTIVITY

Locomotory activity was measured by an automatic recorder (Jones, 1955). The swimming of the fish disturbed a celluloid vane suspended in the water by a work-hardened resistance wire. The movements of the wire completed or broke a circuit, and the number of times that this occurred was automatically counted and recorded on a rotating kymograph drum. The activity of the fish was determined from the number of makes and breaks each hour or half hour. The fish were kept in a glass tank placed in an easterly window well exposed to daylight, and at night to the much lower intensities from street lighting and occasional road traffic. The tank was surrounded by black paper so that the fish would not be disturbed by seeing anyone enter the room. To prevent sudden temperature rises in the summer months water was circulated through the tank, and on very hot days it was shaded from direct sunlight. The rate of flow was so adjusted as to be without effect on the activity

recorder. In some experiments the water temperature was recorded continuously by a thermograph, and measurements of light intensity were made with an R.C.A. 931 A photo-multiplier (Jones, 1955).

Experiments were made with individuals or groups of two to eight fish and lasted from 2 to 10 days, and were carried out over a period of 18 months so as to cover the complete reproductive cycle. During each experiment food was usually provided by a stock of living *Tubifex*. Six series of experiments were made, the conditions of each being as follows:

(1) Cover available in the tank, provided by two small hollow bricks in which the fish could hide.
(2) No cover available.
(3) No cover at first, bricks added later.
(4) Cover available, tank later blacked out by light-proof box.
(5) No cover, tank later blacked out by light-proof box.
(6) Fish blinded by removal of eyes, no cover available.

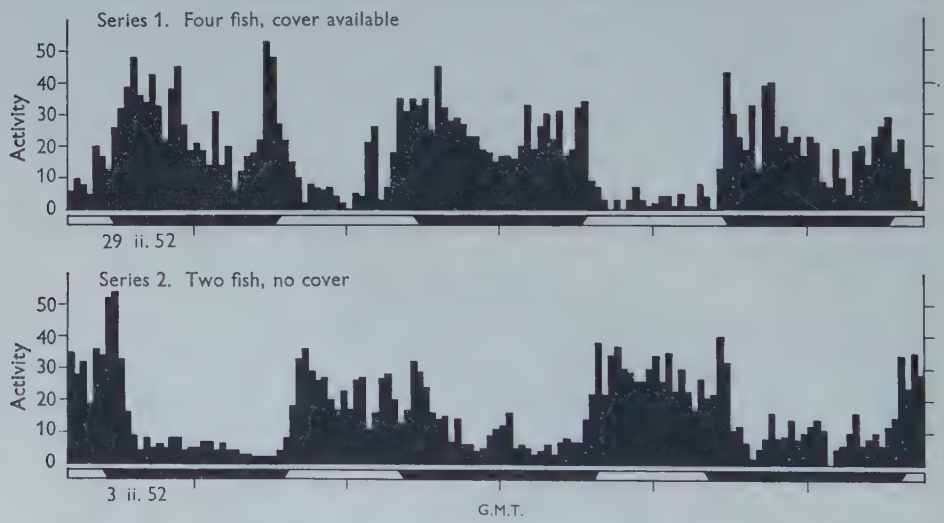

Fig. 1. The activity of minnows with and without cover in which they can hide.

Results typical for each series of experiments are given in Figs. 1–5. When cover was available (Fig. 1, Ser. 1) the fish were more active at night than during the day. At sunset there was a burst of activity which declined during the night to be followed by a second burst at sunrise. When there was no cover (Fig. 1, Ser. 2) the pattern of activity was reversed, activity being greatest during the day, but dawn and dusk peaks are not obvious, although there were suggestions of them in some records. The high level of activity recorded during the day was not due to the fish schooling with their reflexions on the side of the tank, as it was still recorded when the inner surface was painted matt black or when an earthenware crock with an unglazed inner surface was used in its place. The activity of the minnows in these two series of

experiments is related to changes in light intensity (Fig. 2), while the third series (Fig. 3) shows that the pattern can be reversed by altering the experimental conditions. Individuals or groups gave the same results, and the presence or absence of food appeared to have no effect; experiments were not made with starved fish. From April to June, during the breeding season, the minnows failed to respond to the changes in light intensity. They never took to cover during the day when it was available, and their level of activity was generally very high. The fourth and fifth series of experiments (Fig. 4) failed to reveal any trace of an inherent or endogenous rhythm of locomotory activity. Similar negative results were obtained when fish were taken from the stock aquarium and kept in the dark in a sound-proof room for several days.

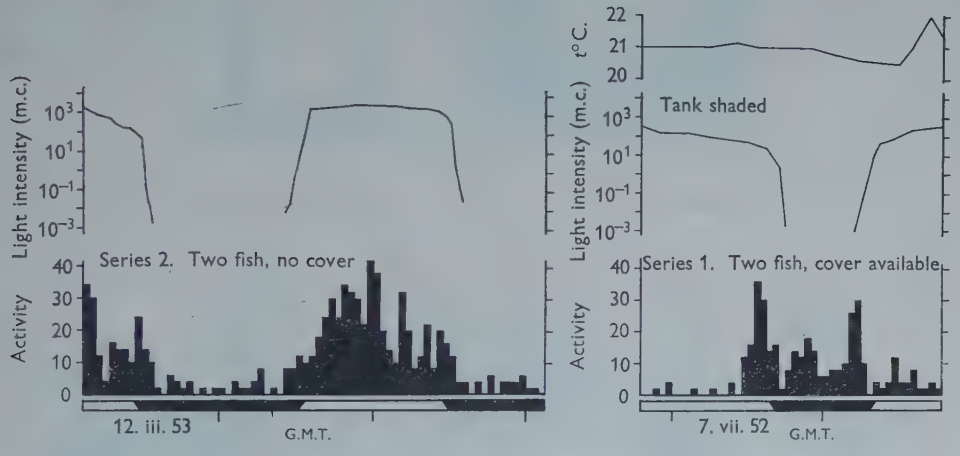

Fig. 2. The activity of minnows in relation to light intensity in the first two series of experiments.

After sunset, the blind fish (Fig. 5) showed a great increase in activity, which declined during the night. There is a suggestion of an increase at dawn, after which the activity remained low throughout the day. The pattern of activity does not appear to bear any relation to temperature. In the record shown in Fig. 5 the rhythm weakened towards the end of the experiment. In two of the blind minnows the melanophores were expanded, as is usual, but in the third they were contracted and the fish was pale. When experiments were made with the fish individually, the pale fish gave the most clear and consistent records of low daytime activity followed by an increase at sunset, and possibly at sunrise, with a return to the day level.

When cover was available the minnows hid in the hollow bricks during the day. Sometimes they came out for a few minutes, but they only appeared to swim freely in the tank at night. Daytime observations showed that the fish usually remained just inside the bricks, occasionally moving forward until the head and eyes cleared the shade, pausing and then backing into cover again. The fish appeared to be making a comparison between the intensities in the brightly lit tank and the shade of the hollow bricks. Their emergence from cover at night and when the tank was blacked out (Fig. 4) suggests that they avoid bright light, and the peaks of activity at sunrise

and sunset may be in response to the rate of change of light intensity which is of the order of 1 log unit in 15 min. However, in one instance, shown in Fig. 2, the peak occurred before any rapid fall in intensity. In one experiment, measurements were made of the light intensity at which the minnows emerged from and retired to cover.

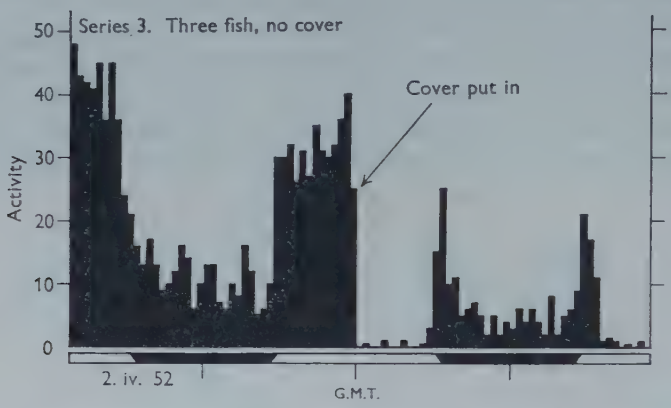

Fig. 3. The change in the pattern of activity when cover was put in the tank.

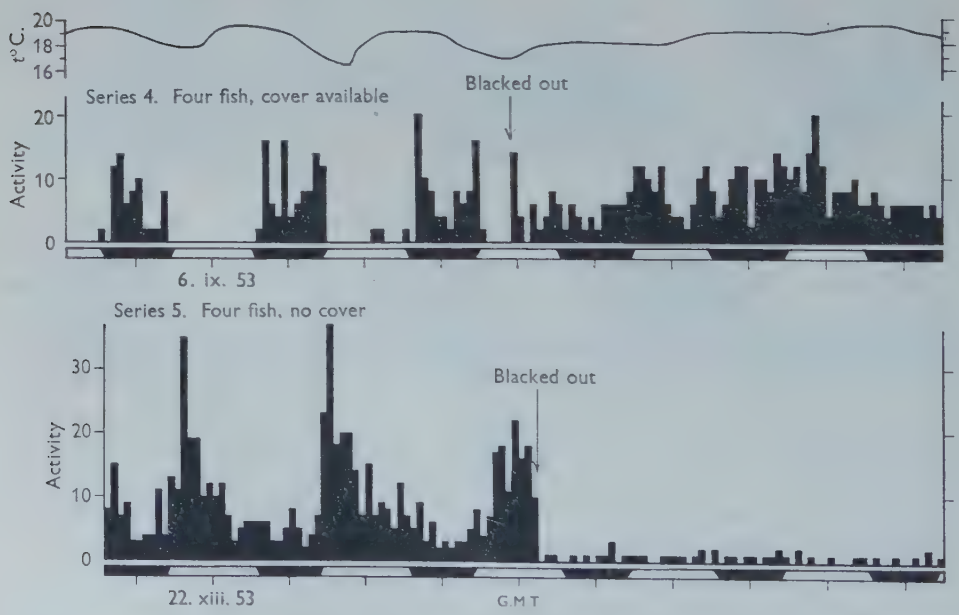

Fig. 4. The effect of constant darkness on the pattern of activity.

On three successive nights, intensities of 1·8, 5 and 55 m.c. were recorded for emergence and 68, 78 and 4·6 m.c. for retiring.

The low night and high day activity recorded when no cover was available, and the sudden drop in activity when the tank was blacked out (Fig. 4), could be the result of a photokinesis which might, under natural conditions, be part of a

mechanism to ensure that they reach cover of lower intensities. Once cover is reached, however, it seems likely that they would remain there by a comparison of intensities, provided the light-dark boundary or gradient was sharp enough.

While no inherent rhythm of locomotory activity was found in minnows kept in continuous darkness, Spencer (1939) found that goldfish had a diurnal activity rhythm which persisted, although weakening, for several days in continuous light. On the other hand, Harder & Hempel (1954) found that the normal activity rhythm of plaice was broken by exposure to continuous light. While slight variations in the melanophore index of minnows persist in the dark (Pauli, 1926), and the movements of the rods and cones of *Ameiurus nebulosus* can be detected for at least 2 days under similar conditions (Welsh & Osborn, 1937), melanophores and rods and cones remain in the light-adapted phase under continuous light. Clausen's (1936) results on the oxygen consumption of the black bass, *Huro salmoides*, are sometimes

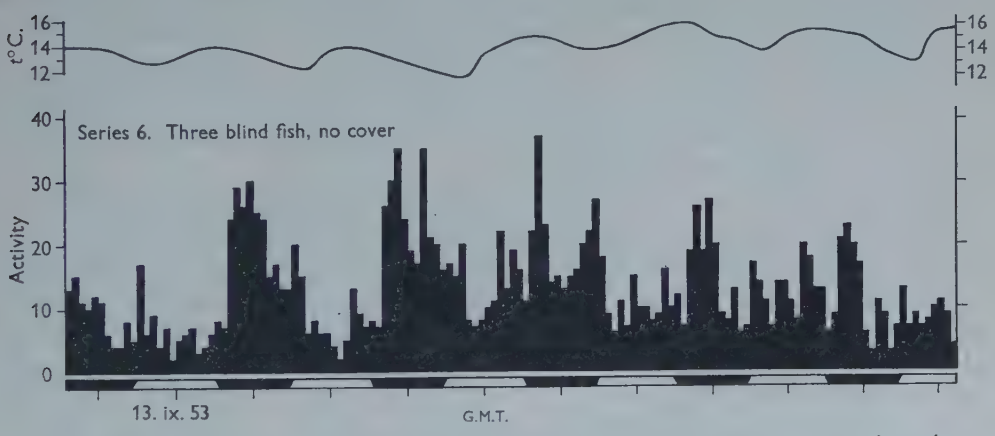

Fig. 5. The response of a group of three blind minnows to normal variations in light intensity.

quoted as demonstrating the persistence of metabolic activity under constant environmental conditions. But as his fish were kept in chambers covered by a cloth 'loose enough around the upper end of the chamber to allow an inspection for air bubbles' it is possible that they could have been stimulated by variations in light intensity.

It was not surprising to find that blind minnows react to diurnal variations in light intensity as their pineal organs and adjacent regions of the mid-brain are light sensitive (von Frisch, 1911; Scharrer, 1928). Their reactions, however, are the reverse of the reactions of normal fish without cover, and it is interesting to note that while the response of naturally blind cave-dwelling characins is dependent on the degree to which the pineal area is exposed to light, removal of the apparently functionless optic cysts makes them indifferent to light (Breder & Rasquin, 1947). Removal of the eyes in minnows might reduce their overall sensitivity to light, which would account for their low activity during the day, while the increase in activity at sunset, and perhaps at sunrise, suggests that the pineal complex is particularly sensitive to changes in light intensity. If this were so, a fish whose melanophores

were contracted, so increasing the exposure of the pineal area, might be expected to respond better than a fish whose melanophores were dispersed, as was found to be the case. But clearly more experimental work is needed here.

REACTIONS AT A LIGHT-DARK BOUNDARY AND SHOALING BEHAVIOUR

The results of the experiments on locomotory activity suggested that minnows avoid high light intensities, and observations were therefore made on their reactions at a light-dark boundary to determine the value of the upper limit. A large aquarium tank, 120 cm. in length, 60 cm. wide and 45 cm. deep, filled with water to a depth of 15 cm., was used in these experiments, which were carried out in a sound-proof and light-proof room. Half the tank was roofed and curtained to the water surface. A 100 W. lamp was suspended 1 m. above the middle of the tank and its intensity was varied by an autotransformer. Measurements of light intensity were made with a neon discharge tube photometer, similar to that described by Poole & Poole (1930). The photometer was calibrated against a uniplanar tungsten filament substandard lamp. Several experiments were made with five to six minnows in the tank which were provided with *Tubifex* as food.

In bright light the minnows remained in the shaded half of the tank, up against the far wall, and never approached the light-dark boundary. When the light intensity was very slowly reduced, the fish swam, in a group, more freely in the shaded half of the tank, but were very hesitant on approaching the boundary and never crossed over while the intensity of the light falling on the water surface in the unshaded half of the tank was greater than 0·17–0·08 m.c. Even then there was some hesitation on approaching the boundary, and it was not until the light was reduced to 0·024 m.c. that they swam freely from one half to the other. At intensities of this order the minnows no longer shoaled together as they did in bright light and further observations were therefore made to determine the intensity at which the shoal broke up. For these experiments the roof and curtain were removed. Shoaling behaviour was clear at 0·08 m.c., but at lower intensities the fish became more and more independent of one another, and the shoal appeared to break up at intensities between 0·024 and 0·0034 m.c. At lower intensities the minnows were very active and excited, breaking the surface as if feeding. They could be heard breaking the surface when the light was too low for observation, but after several minutes this stopped. When the light was turned up, the fish were seen to be resting, independently of one another, on the bottom.

While the method for reducing the light intensity was not entirely satisfactory as there was a shift to the red in the spectral composition of the light as the voltage was reduced, the results suggest that minnows avoid intensities greater than 0·17–0·08 m.c., and that their shoaling behaviour breaks down at intensities between 0·024 and 0·0034 m.c. While it is well known that shoaling behaviour is mainly dependent on vision (Morrow, 1948), and that shoals break up at night, no measurements are available to compare with the present results. Feeding is another activity

which, in the minnow and other fish which actively catch their food, could depend on vision and might be related to light intensity. Herring, for instance, are not filter feeders but catch their prey (Hardy, 1924) and are said to be able to feed at intensities of the order of moonlight, but not by starlight or in the dark (Můzinić, 1931; Battle, Huntsman, Jeffers, Johnson & McNair, 1936; Johnson, 1939). Verheigen (1953) found that herring shoals break up gradually with decreasing light intensity, but that feeding continues at lower intensities; when feeding stops, the herrings are still able to avoid obstacles. To complete the picture for the minnow, experiments were therefore made to determine what effect light had on their feeding ability.

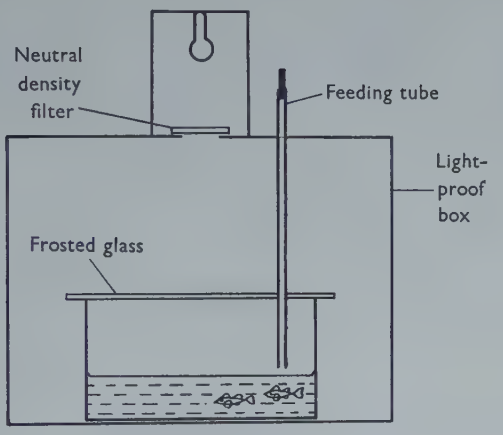

Fig. 6. Apparatus used in the feeding experiments.

LIGHT INTENSITY AND FEEDING

Using the apparatus shown in Fig. 6, experiments were made to see how many of fifty living *Daphnia* two minnows could catch in half an hour at different light intensities. The lower light intensities were obtained by using a torch bulb and neutral density filters and the highest by using a 60 W. lamp. Control experiments were made in the dark. The two minnows used in each experiment were drawn at random from a stock of fourteen fish which had been fed only a few *Daphnia* each day for a fortnight before the series commenced. After the minnows had been left in the light-proof box for half an hour to become adapted to the conditions of the particular experiment, fifty *Daphnia* were added through the feeding tube. Half an hour later the box was opened, the fish quickly removed and the remaining *Daphnia* recovered and counted.

The results are summarized in Table 1 and shown graphically in Fig. 7. Over 90 % of the *Daphnia* were eaten at intensities greater than 0·008 m.c. The minnows did not do so well at lower intensities, but they took 40 % of the *Daphnia* in the dark.

The results show that minnows depend in part on vision for feeding and that the change from visual to 'dark' feeding takes place between 0·0007 and 0·00007 m.c. No attempt was made to find out how the *Daphnia* were captured in the dark.

Table 1. *Number of fifty* Daphnia *eaten by two minnows at different light intensities*

Light intensity in metre-candles	30	0·008	0·0007	0·00007	0·000007	Dark
No. of experiments	7	10	10	14	9	9
Mean number of *Daphnia* eaten	48	46	40	26	25	20
Standard deviation	2·6	4·7	8·9	9·0	7·3	8·6

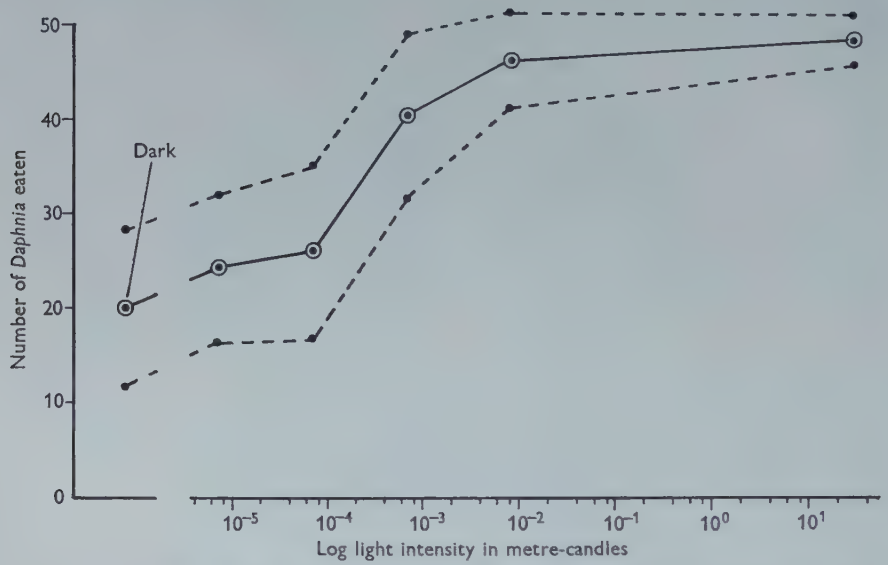

Fig. 7. Number of *Daphnia* eaten by two minnows adapted to various light intensities. Fifty *Daphnia* offered.

DISCUSSION

The results are summarized in Fig. 8, together with values of various light intensities found under natural conditions, and some results of other authors which fit in with the general picture. As judged by their behaviour in the light-dark boundary experiments, minnows avoid intensities greater than 0·17 m.c.; a shoal would break up at an intensity a little below that of moonlight, and they would just be able to feed by eye at the surface under starlight. The change from visual to 'dark' feeding between intensities of 0·0007 and ·00007 m.c. may be related to the change from cone to rod vision, which Brunner (1935) concludes takes place at 0·008–0·002 m.c. But it should be noted that her results with the minnow differ from those of Wolf & Zerrahn-Wolf (1936) with the sunfish *Lepomis*. For this fish it appears that cone vision changes to rod vision at 0·4 m.c., which is about the same intensity at which the change takes place in man.

Minnows appear to keep below their upper limit of light intensity by a comparison of intensities, that is, by a taxis, but the results of the experiments on locomotory

activity suggest that they also respond to light photokinetically. Evidence of a photokinesis in teleosts has been given by Shaw, Escobar & Baldwin (1938), who found that the swimming speed of goldfish fully adapted to intensities of 30 and 550 m.c. is about twice that observed when the fish are adapted to intensities less than 0·5 m.c. Similarly, Schlagel & Breder (1947) found that blind cave characins consume more oxygen in the light than the dark, and Woodhead & Woodhead (1955) have found a photokinesis in larval herring. As a comparison of intensities can only be made if the light-dark boundary is sharp enough, it would be interesting to see how minnows behave in a light gradient of gentle slope and whether they keep below their upper limit by a kinesis or a taxis under such conditions.

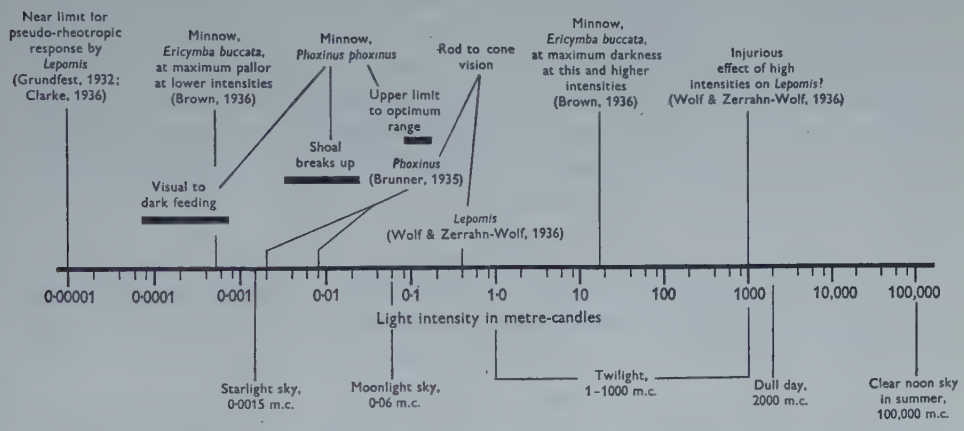

Fig. 8. Diagram summarizing the results obtained with *Phoxinus* with some values of natural light intensities and, for comparison, results of other authors with different species.

If minnows kept below their laboratory limit in the field, they would be expected to hide under stones or weeds during the day, and, if swimming over deep water, to keep below 30 m. during a summer day. In Windermere, Frost (1943) found that minnows live in the littoral zone, hiding during the winter months under stones at depths of 1·5–2 m., and in the late spring, summer and autumn swimming pelagically in depths of 0·3–0·6 m. Under natural conditions then, large shoals of minnows appear to behave quite differently when compared with small groups in the laboratory. However, minnows may not spend all their time in such shallow water as during the summer they mainly feed on cladocerans and copepods which migrate to deeper water during the day (Ullyott, 1938), and the minnows themselves are the principal food of perch over 16·5 cm. in length, which at this time of the year are most abundant at depths of 6–12 m. (Allen, 1935). But in shallow streams there is little doubt that minnows live under light intensities which, under laboratory conditions, they would avoid if cover were available. Under such conditions the minnows would be expected to be very active throughout the day. In the laboratory, complete disregard for light intensity was only shown during the spawning season. Here, at least, is a measure of agreement between the experimental and field observations.

SUMMARY

1. Minnows kept in a tank are active during the day and quiet at night. Their behaviour is reversed if they are given hollow bricks in which they take cover and so avoid bright light. When cover is available they are very active at sunrise and sunset.

2. Minnows have no inherent daily rhythm of locomotory activity.

3. Blind minnows respond to daily variations in light intensity, and are more active at night than during the day.

4. Minnows will not cross a light-dark boundary when the intensity on the light side is greater than 0·17–0·08 m.c.

5. A minnow shoal disperses between intensities of 0·024 and 0·0034 m.c.

6. Minnows catch *Daphnia* better in bright light than in the dark. The change from visual to 'dark' feeding takes place between 0·0007 and 0·00007 m.c.

7. Minnows appear to avoid bright light by a comparison of intensities if the light-dark boundary is sharp, but they may also respond to light photokinetically.

8. It should be noted that these results, obtained in the laboratory, may not be true for minnows under natural conditions.

This work was carried out in the Zoological Laboratory, University of Cambridge, and I wish to thank Prof. Sir James Gray, F.R.S., for letting me work in his department. While the work was carried out I was in receipt of a grant from the Development Commission.

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ELECTRICIAL ACTIVITY IN A SLUG GANGLION IN RELATION TO THE CONCENTRATION OF LOCKE SOLUTION

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In studies of the activity of isolated organs it is desirable to bathe them in a fluid similar to that found in the intact animal. Previous work on nervous systems indicates that their electrical activity is greatly affected by the concentration of ions in the medium, except in cases where they are enclosed in a thick impermeable sheath. In gastropods the suitability of a 0·6–0·75 Locke solution for *in vitro* experiments on isolated organs was demonstrated by Cardot (1921), who studied the effect of different dilutions of mammalian Locke solution on the frequency and amplitude of the heart beat in *Helix*. A 0·7 dilution (sometimes called Cardot's solution) has generally been used by previous workers on the nervous system of gastropods, and their preparations retained constant electrical activity for many hours, but there is an absence of studies on the effect of variations in the composition of the bathing fluid on the gastropod nervous system. This is of interest because the blood of slugs and snails is known to show considerable fluctuations in concentration. The problem may also be of significance in relation to the general behaviour of the animal, since it is known that these fluctuations are related to the activity of the animals.

Thus Duval (1930) and Kamada (1932) found that the body fluid is more concentrated in hibernating and aestivating snails than in active animals. Similarly, Brand (1931) showed that the water content of active snails was substantially greater than that of inactive ones.

Changes in body fluid concentration may occur quite rapidly. Arvanitaki & Cardot (1932) observed a decrease in the depression of freezing-point of the blood from 0·47 to 0·2° C. following rain, and Pusswald (1948) has shown that the major part of the water loss from slugs comes from the blood. She also found that it occurred more rapidly in *Limax* than in *Arion*, and related this to the greater sensitivity of the former to desiccation and its increased activity after rain. In contrast to the findings of these authors, who have assumed a direct relationship between water content and activity, are the conclusions of Wells (1944) and Dainton (1954). Their experiments suggest that high water content is not the immediate cause of activity but that it determines whether a response to a particular sensory stimulus will take place.

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In this paper observations are given on the effect of different dilutions of Locke solution on the frequency of discharge from individual units within the pedal ganglia. The work was made possible by the development of a technique for recording the electrical activity, while the ganglia remained in the bathing fluid for long periods and so were not subjected to drying or oxygen lack.

MATERIAL AND METHODS

Specimens of *Agriolimax reticulatus* were collected from gardens and were usually kept for a few days on damp blotting-paper prior to an experiment. They were fed on pieces of carrot.

The dissection was performed on an unanaesthetized animal pinned to a wax block. The viscera were removed to expose the brain and two pedal nerves running along the foot. The brain consists of two cerebral ganglia (supraoesophageal) and a series of suboesophageal ganglia. The largest of these are the paired pedal ganglia, each giving off a large pedal nerve. In addition, there is a small ring of ganglia

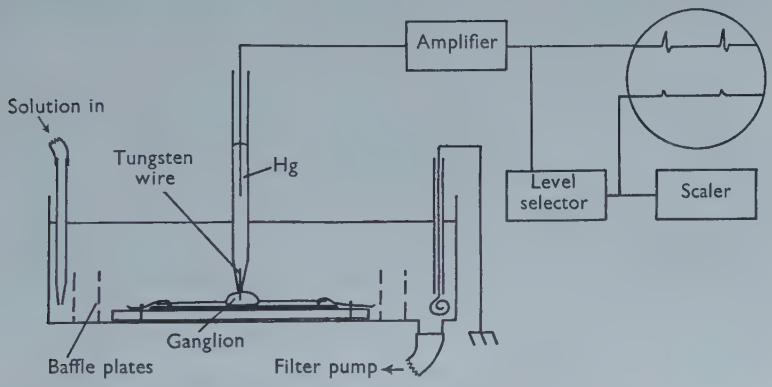

Fig. 1. Diagram of the apparatus used for the continuous counting of the activity of an isolated ganglion. The solution was aerated by a 'bubbler' not shown in the diagram.

situated between the pedal ganglia and the buccal mass. The brain was removed from the animal, together with the long pedal nerves which were ligatured with nylon threads. The preparation was completed by removal of the cerebral and other small ganglia. The pedal ganglia now remained with the two pedal nerves attached to the nylon threads which were used to manipulate the preparation into any required position.

In the first series of experiments the ganglia were hooked beneath chlorided silver wires mounted in a Perspex holder which could be dipped in and out of different solutions.

In the second series the preparation remained in the bathing solution throughout the experiment and did not suffer from periodic drying (Fig. 1). The nylon threads were fastened to a microscope slide by means of elastic bands. A coating of black paraffin wax on the slide made the ganglia more easily visible, and provided a surface which was less likely to damage the fine electrode. The slide was fixed in a

Perspex holder contained in a trough filled with the bathing solution. A series of baffle plates surrounding the slide served to protect the preparation from disturbing currents when the solution was changed. This was done by using a filter pump to drain one solution before the next was run in.

The electrodes consisted of sharpened 0·002 in. tungsten wire fused into a Pyrex glass tube filled with mercury. By means of a Peterfi micromanipulator the electrode was easily thrust into a ganglion. The bathing solution was earthed by a tungsten wire. Impulses were amplified by a Grass P4 amplifier and led to a Cossor double-beam oscilloscope. They were also passed through a level-selecting device before being fed to a Dynatron scaling unit. The input of this was connected to the second beam of the oscilloscope so that it was possible to see exactly which part of the activity was being counted. In some cases negative pulses were counted, but in other preparations the positive part of the wave-form proved more suitable. Readings were taken every 30 sec.

The bathing solutions were made by diluting standard Locke solution; the osmotic pressure of these and other solutions were determined by the depression of freezing-point method of Ramsay (1949).

RESULTS

(1) *The nervous system* in situ

Observations were first made on the electrical activity of the nervous system when the animal was opened and the viscera displaced to one side. The activity recorded from electrodes placed on one of the pedal nerves or adjacent to the pedal ganglia is composed of an apparently random sequence of potentials of varying height and duration (Fig. 2A). Larger spikes occasionally stand out from this background activity. Stimulation of the foot produced a burst of larger waves as did stimulation of the head (Fig. 2B). With the external electrodes it was difficult to detect any definite pattern in the recordings, but a tungsten electrode inserted into the pedal ganglia gave much simpler recordings (Fig. 2E). Rhythmically active units were then readily obtained from almost all the ganglia. The type and frequency of such ganglionic activity were variable, and in no case was there any obvious correlation between the potential changes and movements of the foot, heart, alimentary canal or other parts of the animal. It was possible to modify this electrical activity by stimulating the animals as mentioned above.

(2) *Isolated preparations*

A. *External electrodes*

At the commencement of this work many experiments were carried out using a pair of hooked silver-silver chloride electrodes. As with the *in situ* recordings there was considerable background activity and rhythmically active single units were difficult to distinguish (Fig. 3A). Bullock (1945) obtained similar recordings from the cerebral ganglia of *Agriolimax*. In many preparations well-defined unit activity seemed to be absent, but they frequently showed rhythmical changes in amplitude of the background (Fig. 3B). Such records suggest variations in the degree of

synchronization of several units, such as have been found in other invertebrate ganglia (cf. Arvanitaki, 1942).

Quantitative studies were only carried out on preparations having well-defined unit activity. Although most preparations in air showed several active units, the half-minute count remained relatively constant for periods of up to 8 min. (Fig. 4). It was apparent that after being exposed to air for this time the preparation dried up and the electrical activity was inhibited. This effect was reversible since activity returned when the preparation was immersed once more in Locke solution. In addition to the drying effect, it was found that the removal of the nerves and

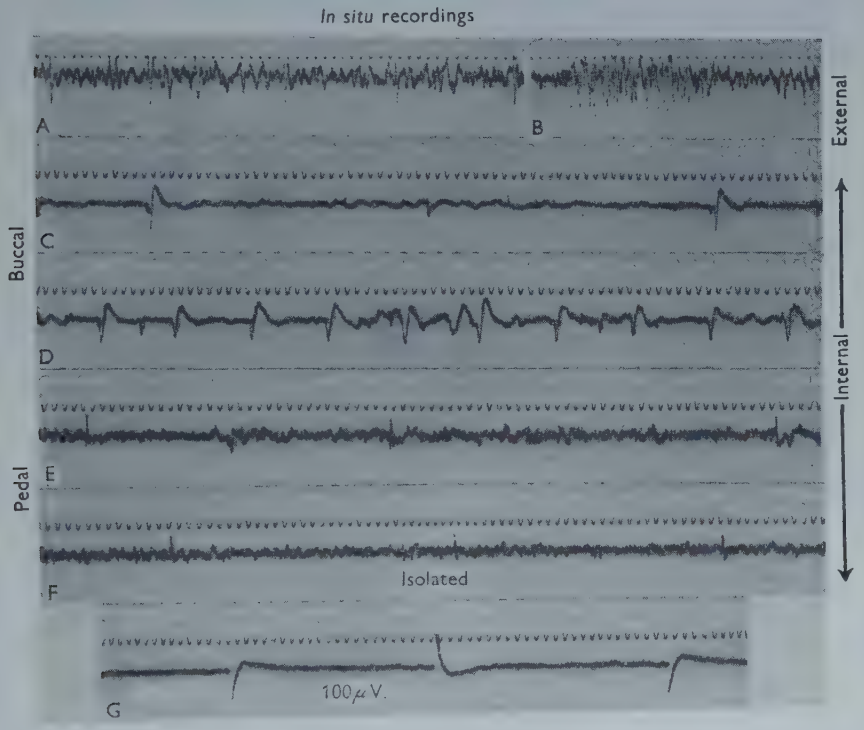

Fig. 2. *In situ* recordings of the electrical activity of slug ganglia. A, external electrode, general activity; B, external electrode, activity after touching the foot; C, internal electrode, buccal ganglion, 2 units active; D, internal electrode, buccal ganglion, many units active; E, internal electrode, pedal ganglion; F, same preparation as E isolated, internal electrode; G, calibration, 100 μV deflexion, time marker 50 cycles.

ganglia from the solution stretched and stimulated the nerves. Drying was overcome to some extent by raising the preparation into liquid paraffin, but the tension at the interface still disturbed the position of the preparation on the electrodes. This was a serious disadvantage for a quantitative study because the size of the potentials recorded from a given unit varied. As this criterion was being used to discriminate between different units, the accuracy of the counts was not constant. Furthermore, the periodic movement of irritable tissue in and out of the solution

must have some stimulating effect and thus decrease the 'spontaneous' nature of the recorded activity. It is apparent that this technique, which has been largely used by previous workers, has many disadvantages if a study is to be made of the effect of variations in the composition of the bathing fluid on the activity of isolated nerve centres.

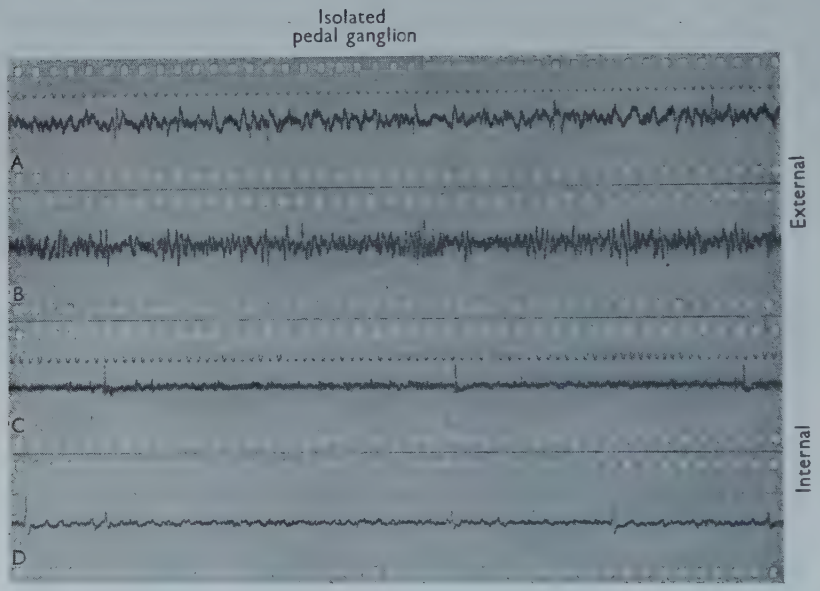

Fig. 3. Recordings of the electrical activity of the isolated pedal ganglion. A and B, externa electrode; C and D, internal electrode. For explanation see text.

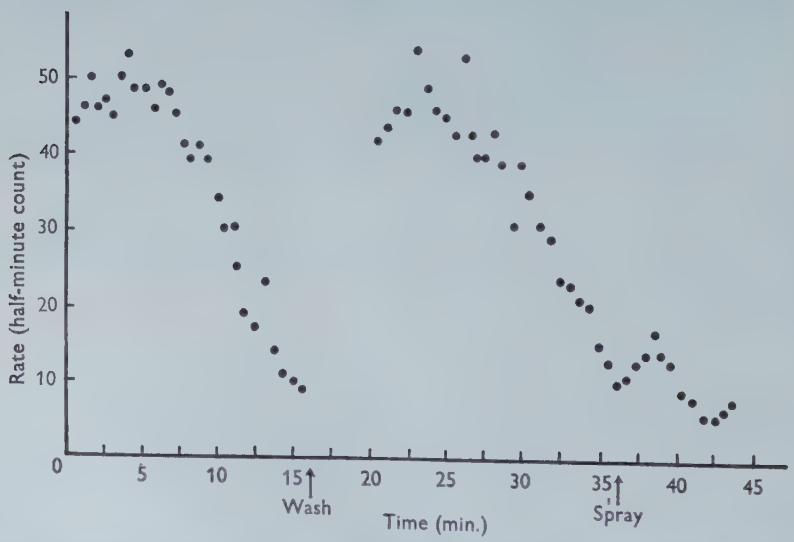

Fig. 4. Effect of drying on the rate of discharge of units in the isolated preparation using external electrodes.

B. *Internal electrodes*

The use of fine tungsten electrodes piercing the sheath surrounding the ganglia largely overcame these difficulties and enabled a study to be made of single units which were easily discriminated from the background (Fig. 3C). By this means a continuous record of ganglionic activity could be obtained over long periods. In many preparations the electrical activity persisted for about 24 hr. The pattern of activity during the first $3\frac{1}{2}$ hr. after isolation is remarkably constant for all preparations (Fig. 5). The half-minute count is high (30) to start with, and usually falls to a lower level (5) after half an hour. Recovery to a count of 10–15 then takes place and this level is maintained with remarkable constancy for about 2 hr. This provides a convenient time during which the effects of changes in the environment can be studied. A steady decline in the frequency of discharge ensues until the preparation dies.

The size, shape and polarity of the potentials varied in different preparations presumably because of differences in electrode position with respect to their source. They were usually triphasic in form and about 200μV. The largest potentials were obtained with the electrode in the centre of the ganglion.

It was rare for there to be more than one active unit recorded, but in cases where this occurred it was noticed that the rhythms were independent of one another (Fig. 3D).

(3) *The effect of variations in the concentration of the bathing fluid*

The same dilution of Locke solution (0·7) used by previous workers on the gastropod nervous system was adopted as the standard solution in the present investigation. The depression of freezing-point of this solution falls in the middle of the range found in the blood of slugs brought in from gardens (Fig. 10) and therefore the tissue was not subjected to harmful osmotic forces during isolation. As has been described above, the pedal ganglion preparation remained in good condition for many hours and shows electrical activity very similar to that recorded *in situ*.

The technique described for changing the solution proved very satisfactory as the change was completed within 30 sec. and the activity of the preparation was not significantly influenced (Fig. 6). Initially, preparations were allowed to equilibrate for 30 min. in 0·7 Locke solution before making any change in the surrounding fluid. As a result of a change in concentration, it was always found that a more dilute solution accelerated the rhythm, whilst a more concentrated solution reduced the activity. The rate of development and magnitude of these effects are roughly proportional to the difference in concentration of the two solutions. Fig. 6 shows the reduction in the half-minute count from 12–14 to 2–3 when the solution surrounding a fresh preparation was changed from 0·7 to 0·8 Locke. This lower level was maintained for 10 min. and immediately rose to about 11 when the original concentration was restored. Similar experiments involving changes from 0·7 Locke to dilutions of 1·0, 0·6, 0·5 and 0·25, were carried out and some of the results shown

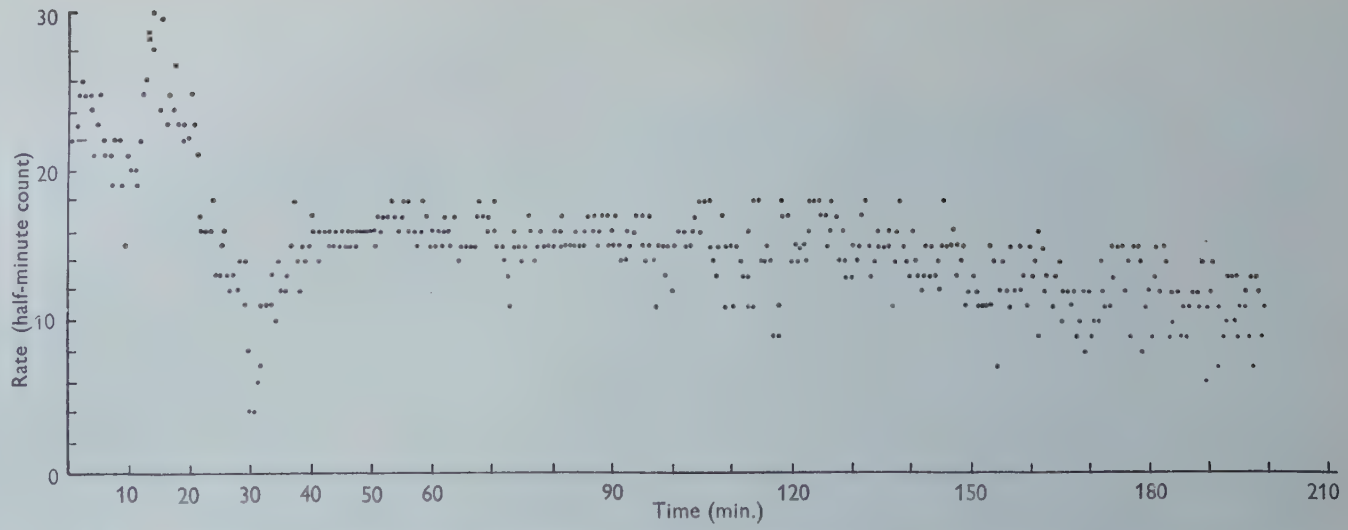

Fig. 5. Typical record of activity of single unit during the first $3\frac{1}{2}$ hr. after isolation. The level of activity in many preparations is less scattered than shown here.

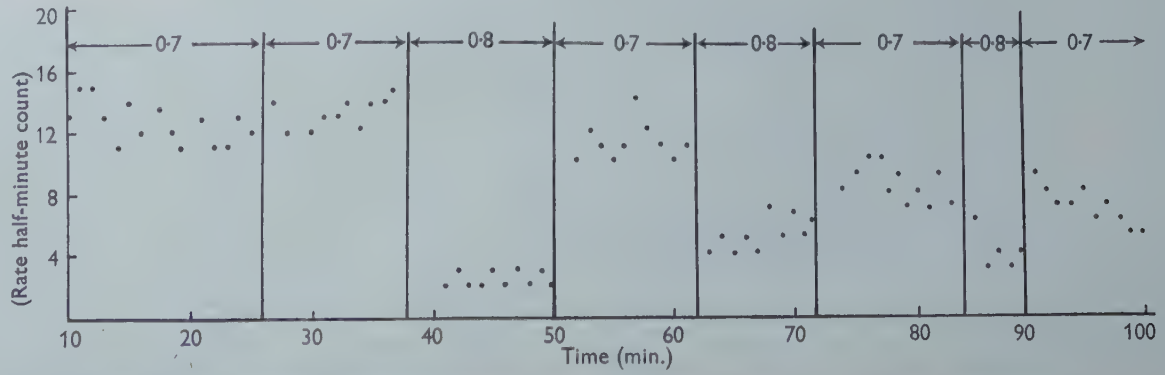

Fig. 6. Effect of an increase in Locke concentration on the activity of a single unit. Note that the change 0·7–0·7 has no effect. The preparation is less active in the more concentrated solution.

in Fig. 7. Most preparations were sensitive to a change of the order of 0·05 Locke, i.e. from 0·7 Locke to 0·65 Locke.

Although in general it is possible to relate the frequency of discharge to the concentration of the medium, this is complicated by several factors. The first of these is the condition of the animal from which the ganglion was isolated. The slugs were kept in fairly standard conditions for some time before the operation, but variations in the nutritive level and/or the state of hydration of the animal (Howes & Wells, 1934) were unavoidable. Arvanitaki & Cardot (1932) experienced similar difficulties when studying the isolated heart of the snail. They found that whereas the heart of one snail might beat perfectly well in a given dilution of Locke, yet the same solution would stop the heart of another snail. Slow acclimatization following isolation allowed both hearts to continue beating normally.

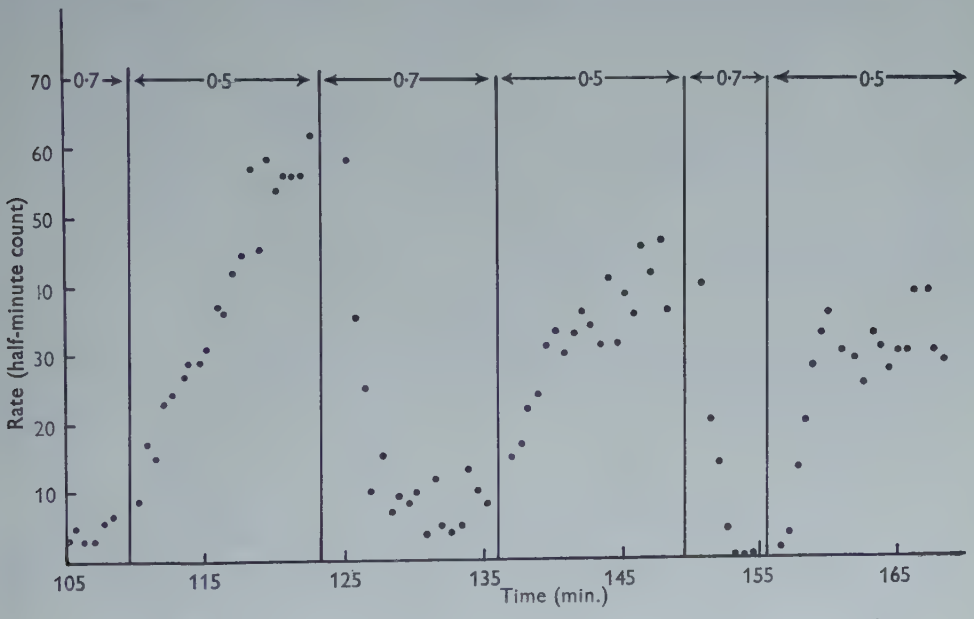

Fig. 7. Effect of a decrease in Locke concentration on the activity of a single unit. The preparation is more active in the more dilute solution.

A second factor concerns alterations in the sensitivity of the preparation which occur with time. In many experiments the first change in concentration produced the most marked effects. Changes in activity brought about by a series of identical changes often became progressively smaller (Fig. 6). This suggests some sort of adaptation similar to Arvanitaki and Cardot's findings with the isolated heart. That the preparation is not completely fatigued by such a series of changes is shown by its response to a more dilute solution after it has become adapted to the change from 0·7 Locke to 0·6 Locke. Such a preparation will still be very responsive to a change to 0·5 Locke.

Further evidence for adaptation is provided by studying the effects of prolonged immersion in a given solution (Fig. 8). It has always been found that the increase

in activity is gradual with a small change in the concentration, and in this case (0·7–0·6) it reaches a maximum about 20 min. after the change, which is unusually slow. There follows a decrease in activity, which is usually maintained above the original level.

It is apparent that nerve cells in the pedal ganglion are sensitive to changes in the concentration of the bathing medium. The precise cause of these changes is not yet clear, but preliminary experiments show that the effect is mainly an osmotic one and not due to the change in the concentration of individual ions. Thus 0·7 Locke solution with mannitol added to make it iso-osmotic with a 1·0 Locke solution had an identical effect on the preparation as a 1·0 Locke solution. Experiments on the pedal ganglion of the snail have given similar results.

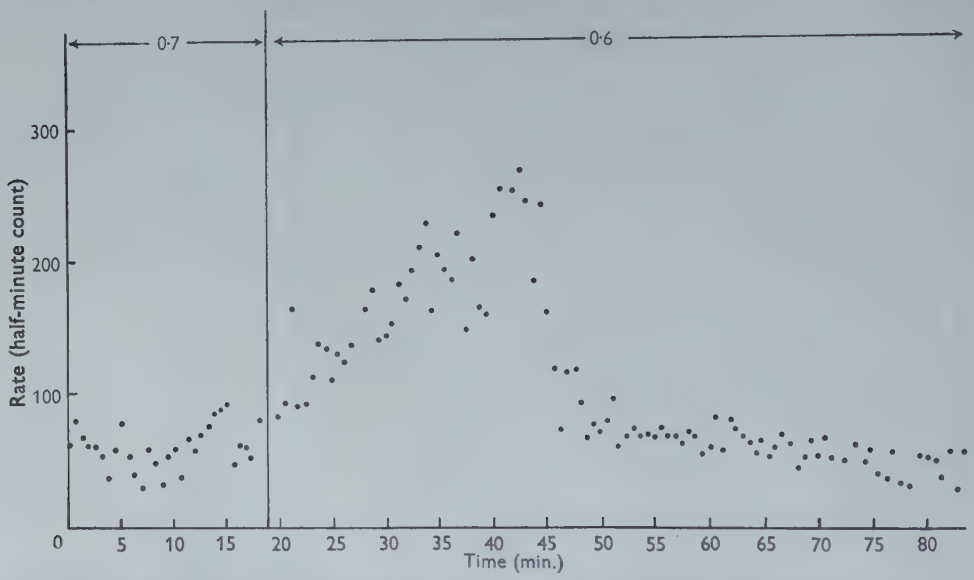

Fig. 8. Prolonged effect of the change from 0·7 to 0·6 Locke solution on the activity of a slug pedal ganglion. The increased activity in the more dilute solution occurred more slowly in this preparation than normally.

(4) *The range in water-content of the slug*

It is desirable to know if the osmotic changes used in these experiments on isolated preparations fall within the physiological range to which the ganglia may be subjected in the life of the animal. Slugs were kept under different conditions of humidity and samples of their body fluid taken. Those kept in very dry conditions soon 'set' as rigid jellies and were completely immobile. Animals dehydrated in a desiccator lost about 35 % of their weight in 3 hr. When slugs treated in this way were allowed to come directly into contact with water, they regained their initial weight within an hour. No change in weight took place if they were kept in an atmosphere saturated with water, but continued desiccation resulted in further loss in weight (Fig. 9). These changes in weight were due to evaporation and not to loss of slime, as the animals were immobile.

During the life of the animal there will be a certain amount of water-loss due to evaporation, particularly in relation to the seasons, but losses consequent on the secretion of slime during locomotion (Kunkel, 1916) probably affect the animal even more markedly. Dainton (1954) found that after 49 min. locomotion in a saturated atmosphere there was a 17% loss in weight and the slug showed reduced activity

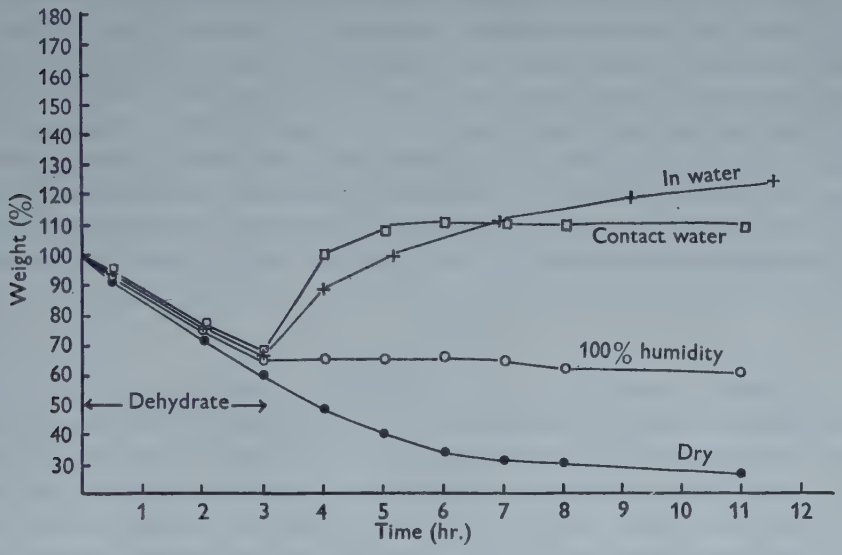

Fig. 9. Decrease in the weight of slugs produced by 3 hr. desiccation. Subsequent changes in weight were dependent upon the conditions as shown.

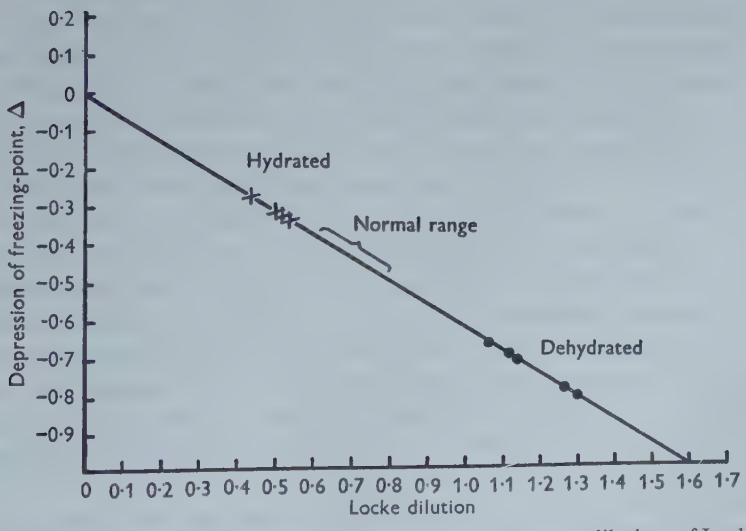

Fig. 10. Straight line giving the depression of freezing-point of different dilutions of Locke solutions. The depression of freezing-point of the body fluids of 'hydrated' and 'dehydrated' slugs is shown in relation to the equivalent dilution of Locke solution. The body fluid of slugs taken from gardens has a depression of freezing-point close to that of 0·7 Locke solution.

in response to further stimulation. A similar decrease in activity followed water-loss due to evaporation. In both cases the greatest fall in water content would occur in the body fluid (Pusswald, 1948) which bathes the nervous system.

Animals maintained in moister conditions showed much greater activity and were more reactive to mechanical and other forms of stimulation. Blood was obtained from these active animals and values for the depression of freezing-point were about 0·3° C. under very moist conditions. The blood of desiccated slugs had a much higher osmotic pressure ($\Delta = 0{\cdot}8°$ C.). Determinations of the osmotic pressure of a range of dilutions of Locke solutions shows that this range is equivalent in depression of freezing-point to dilutions of 0·4 to 1·4 Locke solution (Fig. 10). There appears to be little doubt that changes in osmotic pressure of the body fluids of the slug occur which are comparable in magnitude with those used in the experiments on the isolated ganglia. However, it must be remembered that in the present series of experiments these changes have been relatively rapid, whereas in nature they would presumably occur more slowly.

DISCUSSION

The persistence of electrical activity in parts of the central nervous system when isolated from the rest of the animal appears to be a property of the nervous system of all groups that have so far been investigated. The interpretation of such activity and its significance in the behaviour of intact organisms continues to present problems at all levels of study in the attempt to bridge the gap between behavioural studies and neurological experiments. A major difficulty is to preserve the isolated nervous tissue in conditions as normal as possible. The disruption of the blood or tracheal supply inevitably leads to conditions of oxygen lack in the nerve cells and hence to abnormal activity. Desiccation during the course of an experiment also produces abnormalities. *A priori* it would seem that these dangers are less severe in a study using nervous tissue which is normally bathed in a haemocoel. Such ganglia when isolated in a solution similar to the haemocoelic fluid can be expected to remain in fairly normal or even in unusually favourable conditions with regard to the oxygen concentration. There remain of course the injury effects produced by the severance of the nerves, but these are unavoidable in any isolation experiment.

From the experiments described in this paper it appears that a 0·7 Locke solution is suitable for long-term observations, as activity and excitability persist for over 24 hr. Since similar potentials were recorded in the ganglia before isolation it is probable that the activity studied occurs normally in the slug and is not an artifact due to isolation. Furthermore, as the blood of slugs normally shows fluctuations in water-content comparable with those described in these experiments, it seems likely that the activity of the ganglia *in vivo* will vary in a way similar to that found in the present work.

The potentials recorded represent some unit of activity within the ganglia as they maintain a constant shape and size and are distinguishable from other units by these characteristics. On the whole it was rare to find more than one unit active with a given electrode position. The potentials are probably produced by motor neurones

as each beat is succeeded by a corresponding action potential in the pedal nerve. Whether they are derived from a single cell or from a group of cells cannot be decided, although the smoothness of outline and constancy of shape argue in favour of a single cell. It is significant to note that there are large cells in the pedal ganglia of up to 150μ in diameter. These are comparable with the large cells isolated and examined by Arvanitaki (1942) from *Aplysia* ganglia.

As yet the precise function of this activity in the life of the animal has not been determined. In no case has there been any suggestion of a correlation between contraction of the foot musculature (or of any other activity of the slug) and the rhythmic discharge of these units within the central nervous system. The lack of a direct relationship between impulses in the pedal nerve and movement of the foot was also observed by Turner & Nevius (1951). The co-ordination mechanism of the molluscan foot is incompletely understood, particularly the relationship between activity of the pedal ganglion and the peripheral nerve net, but it is generally accepted that co-ordinated locomotion requires the presence of the pedal ganglion. Perhaps the rhythmic electrical activity studied here represents the physiological basis of the tonic function of the pedal ganglion which forms a part of the hypothesis of several authors (Jordan, 1918; Herter, 1931).

Although no direct connexion can be established between the electrical activity studied in the present work and the locomotory activity of the animal, it is not impossible that a general connexion exists and that the discharge frequency of these units is indicative of the 'vigilance' or central excitatory state of the ganglia.

If this supposition is correct then from the experiments described in this paper it is probable that an increase in water content will produce greater activity of these centres and so influence the behaviour of the animal. It is not suggested that the changed behaviour is a direct consequence of the altered rhythm of the units, but rather that some properties of the central nervous system are changed in such a way as to make the animal respond more readily to peripheral stimuli. Turner & Nevius do not record any observations on the influence of the 'spontaneous' potentials on synaptic transmission through the pedal ganglion or vice versa. This preparation might be suitable for studies on this general problem of the relationship between sensory input to the central nervous system and its intrinsic activity in determining the pattern of motor activity.

This interpretation is largely in agreement with the most recent observations on the locomotory activity of snails and slugs in relation to water-content. Both Wells (1944) and Dainton (1954) have emphasized that high water-content is not the immediate cause of activity, which they suggest is due to some form of sensory stimulation; but they have also pointed out that these animals are more responsive under conditions of high water-content.

However, before any definite conclusions can be reached about the role of changes in ganglionic activity *in vivo* further work is necessary on the nature of the potentials recorded and also on the relationship of the pedal ganglion and the foot nerve net to locomotory activity of the foot.

SUMMARY

1. A method is described by which the electrical activity of single units in the isolated pedal ganglia of the slug *Agriolimax reticulatus* can be studied quantitatively for many hours. When tungsten microelectrodes were used the results were more simple and less variable than those obtained using external silver-silver chloride electrodes.

2. The activity studied consisted of potentials of characteristic size and shape. Their frequency was usually about 30/min. No correlation was observed between these and any rhythmic activity of the animal.

3. The blood of the slug shows considerable variation in its water-content, ranging from a concentration equivalent to that of a 1·4 Locke solution in dehydrated animals to one equivalent to 0·45 Locke in hydrated animals.

Animals taken straight from a garden in the evening had a body fluid equivalent to 0·7 Locke.

4. The 'spontaneous' activity of the isolated pedal ganglia was greatly affected by a change in the concentration of the bathing solution. Concentration of the medium decreased the activity and dilution increased the activity.

This effect appears to be due to the change in osmotic pressure rather than a change in the concentration of individual ions. The possible significance of these changes in the life of the animal is discussed.

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THE PARADOX OF *MYTILUS* MUSCLE. A NEW INTERPRETATION

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INTRODUCTION

The nervous control of muscle has been studied intensively during recent years, particularly in the case of vertebrate striated muscle. Although smooth muscle is much more difficult to investigate, the recording of electrical activity from it (Bozler, 1938, 1946, 1948) offers the prospect of a successful experimental approach here as well, and recently intracellular recording has been carried out from smooth muscle preparations (Bülbring, 1954, 1955). The principal generalization which has emerged is that all muscle fibres, whether striated or smooth, possess a surface boundary which is in a state of electrical polarization, the outside being positive with respect to the inside. When the fibre is inactive the resting membrane potential has a value of 50–100 mV. In all striated muscles, and in those smooth muscles which have been thoroughly investigated, both phasic and tonic activity is associated with some degree of reduction in the resting potential, whether brief, as in the spike potentials which precede twitches and tetanus, or prolonged, as during contracture. This depolarization is effected spontaneously in specialized rhythmically-contracting muscles like heart and gut muscles (Draper & Weidmann, 1951; Bozler, 1948), but is otherwise the result of nervous activity.

There remain many unsolved problems in this field, but perhaps none is more paradoxical than that of the tension control in the anterior byssus retractor muscle (A.B.R.M.) of *Mytilus edulis*. Winton (1937) showed that the muscle relaxes very slowly after it has been induced to contract by continuous direct-current (d.c.) stimulation and quite rapidly after stimulation by alternating current (a.c.). He described the slow relaxation non-committally as being due to an increase in muscle 'viscosity' produced by this particular kind of d.c. stimulus. Nevertheless, the implication was that the d.c. stimulus has not only brought about a phasic contraction, but also altered the molecular configuration of the contractile elements in such a way that the resistance to stretch is greatly increased. 'Relaxation' is accompanied by a very gradual return to the much lower resting 'viscosity'. Winton also found that on applying an a.c. stimulus to the muscle during the phase of high 'viscosity' the resulting additional contraction was followed by relaxation at the much higher speed normally observed after using a.c. alone. The a.c. had produced a small phasic contraction and abolished the state of high 'viscosity' at the same time. These results were confirmed by many subsequent investigations (Fletcher, 1937*a–c*; Nieuwenhoven, 1947; Singh, 1938*a*, *b*; Twarog, 1954; Johnson, 1955). Fletcher

showed, in addition, that short-duration d.c. pulses act on the muscle in a manner similar to a.c.

All these authors have taken the view that the results of their experiments support the 'catch mechanism' hypothesis which maintains that molluscan smooth muscles possess a special tonic contractile mechanism where the molecular elements 'set' or 'catch' in the shortened state, thus enabling the muscle to maintain a tonus without doing work (cf. von Uexküll, 1929). Against this view Ritchie (1928), Bozler (1948) and Lowy (1953) have argued that tonic contraction in these muscles is really due to a tetanus. But in the A.B.R.M. no electrical activity has so far been found which would have supported an explanation of its behaviour in terms of the tetanus hypothesis. Fletcher (1937) made an extensive study of this muscle, and reported that although he could detect a large propagated action potential following single shocks to the isolated muscle (each shock resulting in a small twitch) there was no electrical activity at all during prolonged contractions induced by a d.c. stimulus.

On modern views the tetanus hypothesis requires that periodic depolarization of the muscle membranes must occur in order that contraction may be maintained. This should be set up by nervous activity, although myogenic action (i.e. spontaneous depolarization as of heart muscle) would be regarded as equivalent. The 'catch mechanism' hypothesis, which reached its most extensive speculations in the thesis by Nieuwenhoven (1947), requires only the set of events occurring at excitation to bring about the state of maintained contraction which is equivalent to Winton's state of high 'viscosity'. Special conditions are required to bring such a tonic contraction to an end quickly. In the A.B.R.M. preparation relaxation times have been found to vary from 20 sec. to as long as 4 hr. On the tetanus hypothesis relaxation occurs automatically when the postulated continuous excitation diminishes or disappears—a process in conformity with the evidence about relaxation of striated muscle (Hill, 1950). It is, on the other hand, a necessary corollary of the 'catch' mechanism hypothesis that any sort of rapid relaxation such as can be observed after treatment with a.c., various drugs like serotonin, brief pulses and 'weak' stimulation of the pedal ganglia (cf. Nieuwenhoven) must be due to an active process, i.e. a process which results in a reversion of the molecular changes or an undoing of the 'catch'.

One of us has recently used electrical recording technique to demonstrate spontaneous electrical activity in a variety of lamellibranch muscles, particularly in smooth adductors with well-developed tonic properties (Lowy, 1955). These results serve to draw further attention to the *Mytilus* A.B.R.M. preparation where the isolated muscle, cut and tied at both ends, can be made to show all the phenomena of tonic contraction, rapid and slow relaxation, which are also the properties of this muscle in intact animals. One of us (G.H.) has presented this preparation to honours students in 4 successive years and studied it intensively for several months; J.L. has worked with it intermittently for 5 years. In all, a very large number of preparations have been investigated. In many, including those used by students, results similar to those of Winton were obtained, but at least as many gave anomalous results which are worthy of consideration.

Occasionally a student has reported the exact converse of the Winton results, i.e. rapid relaxation following d.c. and a prolonged contraction following a.c. stimulation. Sometimes a burst of a.c. interposed during a tonic contraction due to d.c. has produced a heightening of the contraction with no subsequent relaxation. On two occasions students obtained relaxation of a tonic contraction during the course of applying a weak a.c. stimulus, with a latent period of about 20 sec., but as soon as the weak a.c. was stopped the muscle contracted again to a greater height than that from which it had relaxed, now to continue to relax at the earlier very slow rate.

These observations appeared to throw doubt on the earlier interpretations and a thorough re-investigation of the preparation was decided upon. This has been carried out independently by the two authors but is here reported jointly.

MATERIAL AND METHODS

Animals were kept in fresh aerated sea water before study and opened in the conventional way, i.e. by cutting through the posterior adductor muscle with a knife inserted between the valves. The two halves were gently prised apart, the foot seized with forceps and pulled steadily backwards until it came away completely together with the pedal retractor muscles. This procedure exposes the root of the byssus threads, which are attached to the tendons of the byssus retractor muscles and therefore form a convenient point of attachment for a thread. By cutting out small disks of the shell in the regions to which the anterior ends of the byssus retractor muscles are attached, these muscles can be isolated intact. Following careful 'cleaning' to remove the nerves, connective tissue, etc., a very good preparation can be obtained. It was noticed in a large number of animals that the left A.B.R.M. is divided into two almost equal muscles. One of these is attached to the byssus on its own side and the other to the byssus on the opposite side. This arrangement probably helps to strengthen the base of the byssus.

In view of the fact that Winton and most of the previous authors had used muscles which were cut at both ends, many muscles were treated in this way during the present experiments; some were tested first whilst intact and again after being tied and cut. No serious difference has been observed between the results.

For experiments in which electrical amplification was not required the muscle preparations were mounted in a Palmer muscle-bath and immersed in sea water at about 14° C., contractions of the muscle being recorded isometrically on smoked paper. The muscle was placed so that it ran through a 'knife-edged' slot made in a Perspex strip (Fig. 1). Stimulation was accomplished by passing current across the strip using chlorided silver electrodes. Electrical records were obtained after running all the sea water out of the bath. The recording electrodes were cotton-wicks, soaked in sea water tied on to chlorided silver wires or platinum electrodes coated with platinum black. Condenser-coupled pre-amplifiers with low noise level were used, and electrical activity displayed after further direct-coupled amplification on an ink-writing oscillograph. No special attempt was made to avoid the stimulus artifact, which was considerable with the high gain employed; a shunt

was used to avoid recording it during intensive stimulation. The period of shunting is indicated in the records by a horizontal line under the electrical activity trace. An additional thickening of the line indicates electrical stimulation.

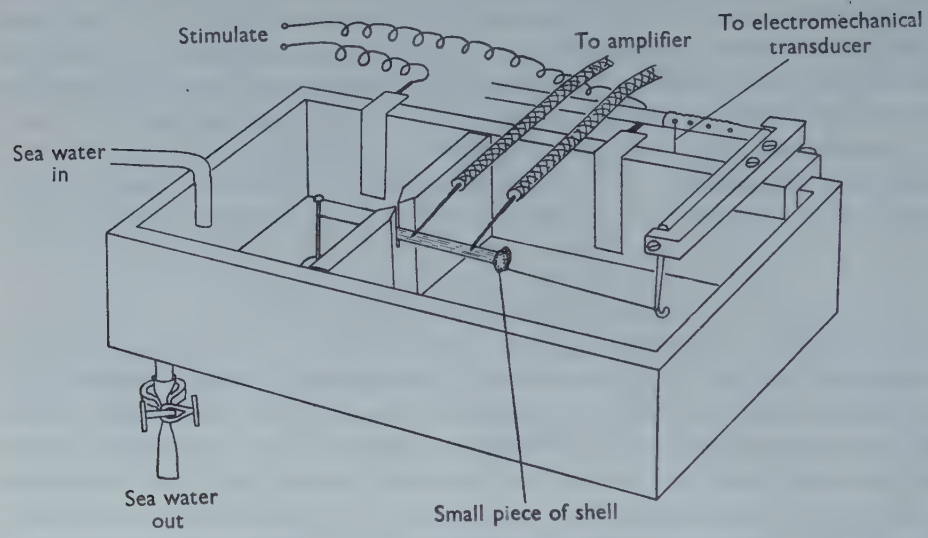

Fig. 1. Diagram of the preparation to show the method of stimulation in sea water.

RESULTS

Direct- and alternating-current stimulation

With the method of stimulation used in the present experiments it is probable that at threshold strength stimulation will occur only at the bridge on the cathodal side. It was found that a direct-current voltage of only 1 or 2 V. applied for 3–4 sec. is usually adequate to produce complete contraction of the muscle. Further stimulation adds nothing to the height, although it frequently increases the subsequent duration of the contraction. When fully contracted the length of the muscle is about a third that of the resting muscle. Winton used more than 10 V. for 10 sec., and even so does not seem to have achieved complete contraction for he obtained a 'staircase' effect with successive stimulations. He was passing current through the whole muscle (immersed in sea water) in an attempt to stimulate it evenly. In the present experiments the character of the response is markedly different from that obtained by Winton. It seemed quite likely that the stimuli were taking effect via the nerve, and this could explain the difference. Such a view is supported by the fact that the low threshold was obtained with the bridge at the byssal end, about 3 mm. from the termination. This is actually the place where the nerves from the pedal ganglia enter the muscle.

If the direction of flow of the stimulating current is now reversed, keeping the bridge close to the byssal end of the muscle, the resulting contraction (even with very large currents) is always minute. This contraction is attributable entirely to the

small length of muscle on the cathodal side. When the current ceases there is now often a marked 'break' contraction involving the rest of the muscle. The height and duration of this contraction are roughly proportional to the strength and duration of the previous stimulation (Fig. 2). It is interesting to recall that anodal break phenomena contributed greatly to the level of contraction in Winton's experiments. If the interpretation of the situation in the present experiments is correct, namely, that excitation is effected via the nerve, the impulses set up in the nerve fibres on the cathodal side of the bridge must be blocked on the anodal side, and the anodal break contraction could be due to repetitive firing in these fibres on removal of the block. Unfortunately it does not seem possible to test this possibility satisfactorily. No matter where the bridge is placed on the relaxed muscle, contraction always occurs on the cathodal side—but it is also fairly complete. In Fletcher's experiments, where cotton wicks carried current to the muscle suspended in air, contraction only occurred in the region of the cathode, and it seemed as though he was stimulating the muscle fibres directly.

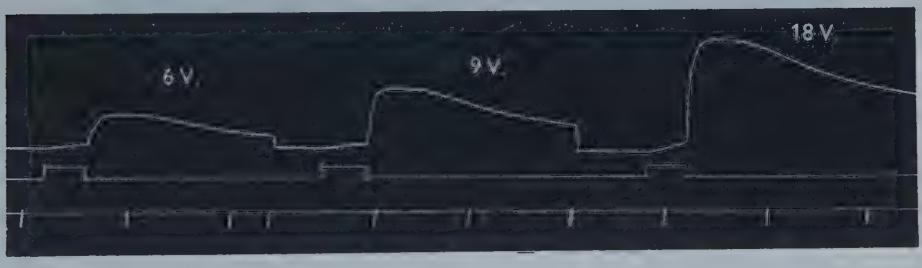

Fig. 2. Anodal break responses obtained with a bridge at the byssal end of the muscle and the major length of the muscle on the anodal side. The drum was stopped between experiments. Time in min.

In spite of the localization of his contraction in the cathodal region (1937*c*, fig. 6), Fletcher claimed that an action potential was propagated along the muscle fibres outwards from the point of stimulation along the whole length of the muscle.

The rise-time of the contraction is fairly constant with d.c., 50~a.c., and a 20/sec. pulse stimulation, being from 15 to 20 sec. Both the shape and the duration of the relaxation curve are, however, extremely variable following direct-current stimulation. At its fastest, relaxation may occur as quickly as the phasic contraction, so that the whole curve of contraction and relaxation occupies less than a minute. At the other extreme very gradual relaxation may take 3 or 4 hr. and complete tonic contractions may last even longer than this. Sometimes there is an initial rapid relaxation, followed by a prolonged period of maintained tonus, followed finally by slow relaxation. All these various results can often be obtained from the same preparation under identical conditions with similar stimulations. It thus appears that in some experiments a separate tonic response is obtained from the preparation, and in others it is not. On the 'catch mechanism' hypothesis it would be argued that on some occasions the 'catch' is set by the stimulation (or after about 30 sec.

latency), whilst in others it is not. Even if this explanation is accepted it does not satisfactorily account for the great variety of the relaxation curves.

Alternating currents of threshold strength applied across the knife-edge bridge produce similar phasic contractions. The voltages required are much less than those used by Winton, but the results are on the whole rather similar. Relaxation usually occurs immediately, and the whole course of contraction and relaxation seldom takes more than 120 sec. Sometimes, however, prolonged tonic contractions are set up by a.c. (Fig. 3). This has happened even in preparations which did not go into a state of tonic contraction after d.c. Such occasional anomalies are most significant, for they rule out the argument which has been developed following Winton's work,

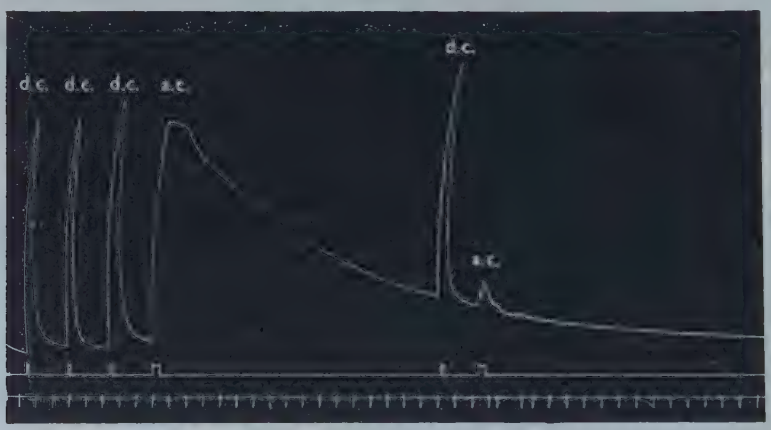

Fig. 3. Anomalous responses to d.c. and a.c. stimulation. Time in min.

namely, that the tonic phenomena, Winton's high 'viscosities', are specially related to the continuous d.c. kind of stimulation, and that the a.c. kind of stimulation leads to a state of low 'viscosity'. The present results of experiments with d.c. and a.c. stimulation show quite clearly that the tonic phenomena must be regarded as special responses of the preparation. They are more readily produced by d.c. stimulation, but there is probably no particular connexion between molecular events in the muscle and the kind of current passing through it. It is possible to divide the responses of the muscle into two separate categories, phasic and tonic. The former is fairly regular in action, while the latter is extremely irregular and requires elucidation.

Inhibition

The presence of inhibitory nerve fibres capable of relaxing tonically-contracted mollusc muscles was first demonstrated by Pavlov in 1885. Nieuwenhoven (1947) showed that the tonically-contracted A.B.R.M. could be made to relax by weak faradic stimulation of the pedal ganglia, thus indicating the possibility of the presence of inhibitory nerves supplying this muscle. Fletcher had shown that under certain conditions electric pulses applied to a tonically-contracted muscle produced relaxation *without evoking any additional contraction* first. The use of the term 'inhibition'

for these relaxation phenomena is not strictly justified in the absence of a complete understanding of the processes involved in contraction. 'Inhibition' actually implies an acceptance of the tetanus theory of tonic contraction. Nieuwenhoven correlated his own and Fletcher's observations, and constructed a hypothesis of an active relaxation process effected by nervous elements. Thus he supposed that tonic contraction involves the operation of a 'catch' and that this state is unlocked by the action of 'inhibitory' nerve fibres running from the pedal ganglia. Subsequently, this view has received support from the work of Twarog (1954) and Barnes (1955). Twarog extracted from *Mytilus* the substance 5-hydroxytryptamine (5-H.T.), a pharmacological agent known as serotonin which raises tonus in vertebrate smooth muscle. She showed that concentrations of this drug as low as 10^{-6} exert a powerful relaxing effect on the tonically-contracted muscle.

With the kind of stimulation used in the present experiments, where it seemed possible that stimulation of the muscle was occurring via the motor nerves, it was reasonable to hope that it would also be feasible to stimulate the postulated 'inhibitory' nerve fibres.

It was found in fact that with the bridge on the point of entry of the nerve from the pedal ganglia weak rectangular pulses, condenser discharges or alternating current can all be used to promote the rapid relaxation of the tonically-contracted muscle. The threshold voltage is approximately half that required to produce contraction. The rate and final extent of relaxation are functions of the duration of the 'inhibitory' stimulation. There is a long latent period of 15–20 sec. before relaxation commences, and it usually continues for a similar period after the stimulation. Hence a burst of 'inhibitory' stimulation may produce an effect only after it ceases.

From this evidence it seems probable that nerves from the pedal ganglia contain fibres which liberate a substance, destroyed or lost by diffusion only slowly, which effects relaxation of the tonically-contracted muscle. The relaxing effect can be obtained at any level of tonic contraction (Fig. 4) and almost any degree of relaxation can be achieved depending on the frequency and duration of stimulation and also to a lesser extent on the strength of stimulation. All these effects are, however, so variable that a quantitative appraisal of the phenomena is not possible. Relaxation can also be produced by certain drugs, e.g. L.S.D.* and 5-H.T., but the rate of relaxation which they bring about (even in high concentrations) is always much slower than that produced by stimulation of the 'inhibitory' nerves. In fact stimulation of the 'inhibitory' nerve accelerates relaxation started by 5-H.T.

5-Hydroxytryptamine

Although Twarog (1954) demonstrated the relaxing action of this drug she did not study the effect of stimulating a treated muscle. This has been done in the present experiments and produced most interesting results. After treatment with 10^{-6} 5-H.T. the muscle, although completely relaxed, nevertheless responds to all kinds of electrical stimulation with a powerful phasic contraction. This is always followed by rapid relaxation (Figs. 5, 7). 5-H.T. therefore abolishes the tonic

* Lysergic acid diethylamide (Sandoz Products).

response without diminishing the phasic one. These findings strongly support the contention made above that phasic and tonic contractions are due to two independent systems.

Following treatment with 5-H.T., muscles were washed at intervals in fresh sea water and subjected to d.c. stimulation. It was found that tonic responses could not be obtained for several hours and in some instances only after 40 hr. Evidently the muscle is unable to destroy the drug, so it seems highly unlikely that it could be identified as the substance naturally responsible for relaxation as Twarog suggested. Following relaxation produced by stimulating the 'inhibitory' nerve, powerful tonic contractions can be produced again immediately.

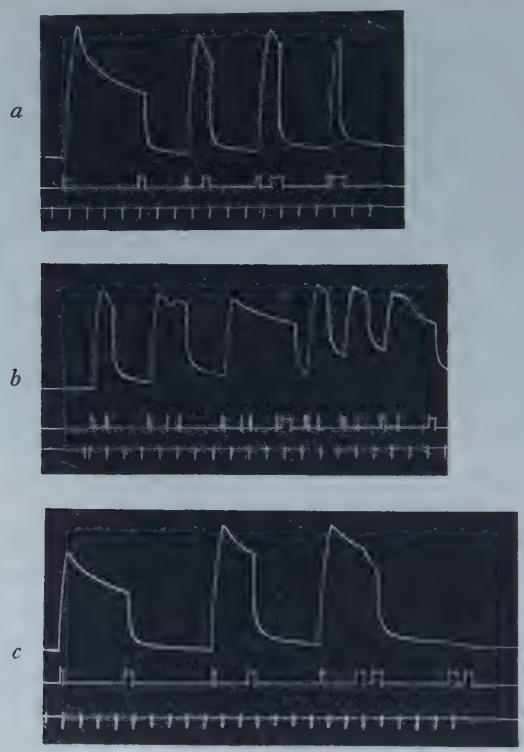

Fig. 4. Records of relaxation (inhibition) of tonic contractions. The contractions were produced by continuous direct current and the relaxations by rectangular pulses, marked by the first and second stimulus marks respectively in each record. Relaxation without preceding contraction could be effected at any stage during the gradual decline of the tonic response. The last two traces of records *b* and *c* show the effects of increased frequency and intensity of inhibitory stimulation. Time in min.

Spontaneous activity

Singh (1943) noted that spontaneous contractions sometimes occur in the isolated A.B.R.M. and they have occasionally been noted by us. Following the rapid relaxation usually obtained after a.c. the muscles often contract again partially, and maintain the new level of contraction by the tonic system (Fig. 6).

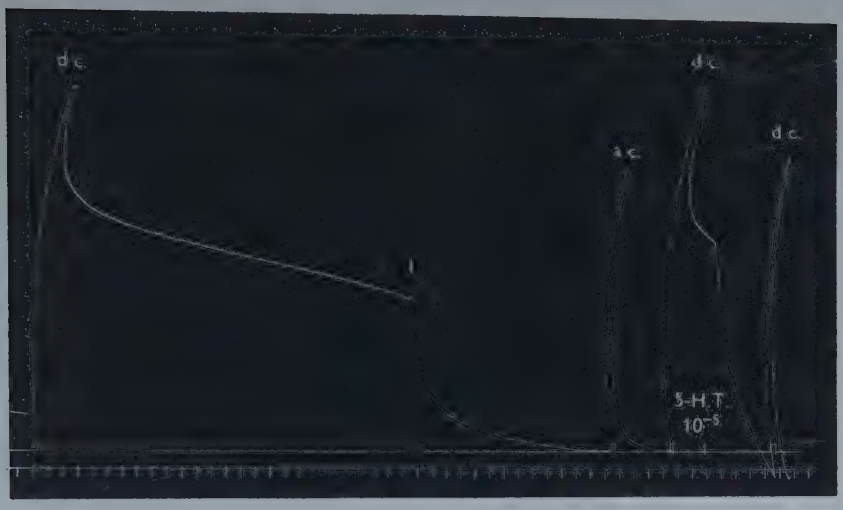

Fig. 5. Record showing: typical tonic response following d.c.; stimulation of inhibitory nerve (at I); phasic response to a.c.; relaxation of a second d.c. tonic response with 10^{-5} 5-H.T. and final phasic response only of the treated muscle to similar d.c. Time in min.

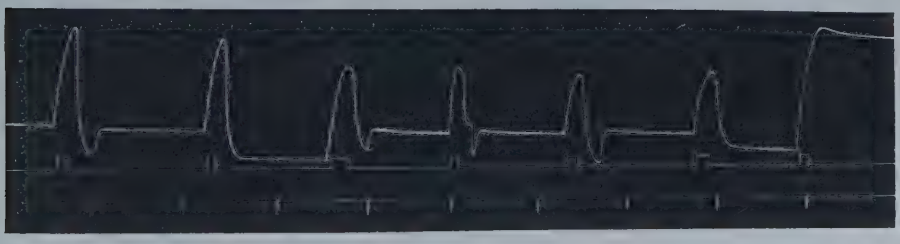

Fig. 6. Spontaneous tonic contractions. The first six responses were evoked by a.c. In four of them relaxation was interrupted by a spontaneous contraction which was maintained by the tonic system. The last response was evoked by d.c. Time in min.

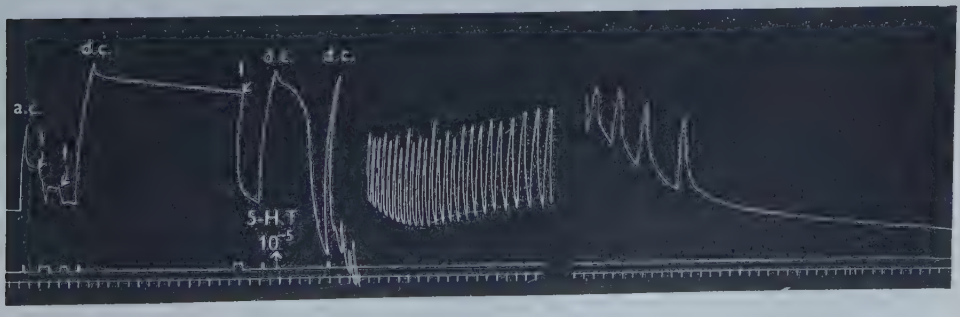

Fig. 7. Spontaneous rhythmic activity. The record shows first: testing inhibitory stimulation; then d.c. stimulation followed by a tonic response which was inhibited at *I*; a second tonic response which was relaxed with 10^{-5} 5-H.T.; phasic response only of the treated muscle but followed by rhythmic phasic contractions. Time in min.

After the tonic response has been abolished by 5-H.T. a direct current stimulus may set up the usual contraction and relaxation, but this is often followed by rhythmic contractions (Fig. 7) which occur at the rate of about 1/min. and can persist for 20–30 min. after which they gradually subside. Further d.c. stimulation is now followed by a resumption of the rhythmical activity (Fig. 7).

Electrical activity

If electrical activity were produced by the *Mytilus* A.B.R.M. as by vertebrate smooth muscles, it might be possible to clarify some of the problems outlined above. There seemed at first little hope of this. Fletcher (1937) had reported electrical activity only in the form of single action potentials following stimulation by a large condenser discharge, with no activity at all during a tonic contraction produced by d.c. But recently one of us (Lowy, 1953) demonstrated that tonic contractions in intact *Mytilus* adductors is accompanied by continuous electrical activity, and it therefore seemed worth while searching for muscle potentials of small magnitude in the isolated A.B.R.M. This effort was amply rewarded.

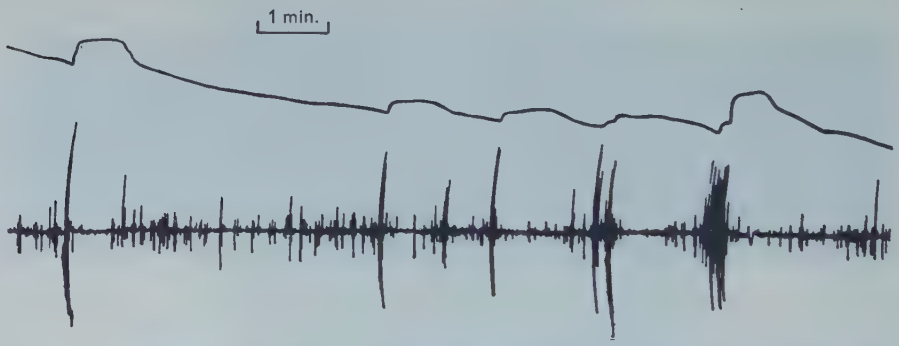

Fig. 8. Simultaneous recording of tension (upper trace) and electrical activity (lower trace) occurring spontaneously in the nearly intact preparation.

It was decided to look first for electrical activity in the intact A.B.R.M. As described earlier (p. 297), the muscle was exposed *in situ* and recording electrodes were placed on it. No action potentials resembling the very large ones described by Fletcher have ever been recorded, but many much smaller potentials were always found to be present (Fig. 8). These potentials appear spontaneously in irregular bursts, each composed of spikes of various amplitudes and of low frequency. The more vigorous bursts are associated with small contractions (Fig. 8). At this stage the dissection was carried a step further and the nerves leading to the muscle from the pedal ganglia were cut. Electrical activity in the muscle did not cease, although after a while it subsided considerably. The final stages of the dissection were now carried out and the muscle treated as an isolated preparation. After soaking in

oxygenated sea water for about 1 hr. an externally denervated muscle prepared in this way shows plenty of spontaneous electrical activity. Other lamellibranch muscles have recently been found to show spontaneous electrical activity following external denervation (Lowy, 1955).

Electrical stimulation

Application of a.c. or of brief rectangular pulses produces a phasic contraction associated with a short outburst of electrical activity (Fig. 9*a*, *b*). In contrast, a d.c. stimulus gives rise to a longer lasting contraction and the electrical activity does not cease when the current is stopped (Fig. 9*c*). Instead, electrical activity continues at a high level for some time (often up to 10 min., depending on the initial duration of the d.c. stimulus), whilst the muscle relaxes slowly (Figs. 10, 11).

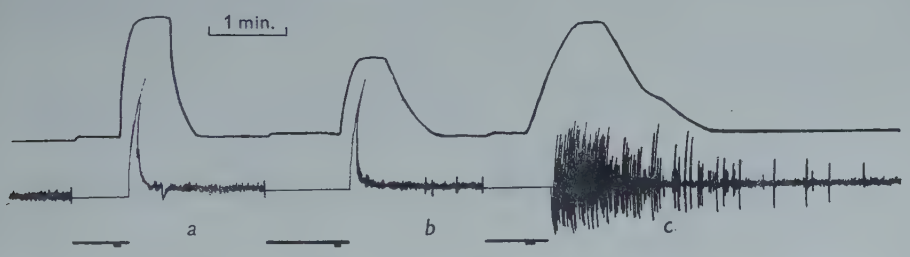

Fig. 9. The mechanical (upper trace) and electrical (lower trace) responses of the A.B.R.M. to: *a*, a.c.; *b*, rectangular pulses; *c*, continuous d.c. The duration of shunting of the amplifier leads is indicated by a horizontal line under the electrical response trace. The duration of stimulation is indicated by a thickening of this line.

In some preparations electrical activity during tonic contraction is very irregular (Fig. 11*b*), and it was discovered at this stage that even a small shift of the recording electrodes could produce a marked change in the pattern of the response recorded: there are sites at which almost no electrical activity of any kind can be detected, although a small shift of the electrodes reveals plenty. In other sites there is complete silence after the initial burst associated with contraction, and instances were observed in which activity occurs only some time after the cessation of the electrical stimulus. Furthermore, electrical activity is often present during relaxation; this will be discussed later. Recordings made using two pairs of similar recording electrodes placed on different regions of the muscle and simultaneously recording reveal that during tonic contraction there is almost no simultaneous or co-ordinated activity in the different regions (Fig. 12).

Drug action

Drops of 10^{-7} acetylcholine applied to the isolated A.B.R.M. give small contractions accompanied by short bursts of potentials. Higher concentrations evoke more powerful contractions accompanied by vigorous electrical activity. There is a

prolonged tonic contraction as Twarog (1954) demonstrated, and the responses to acetylcholine greatly resemble those produced by direct current.

Treatment with 5-hydroxytryptamine effects a marked reduction in electrical activity which normally follows d.c. stimulation (Fig. 13).

Inhibition

When 'inhibitory' stimulation is applied, as described earlier, the spontaneous discharge associated with prolonged tonic contraction disappears.

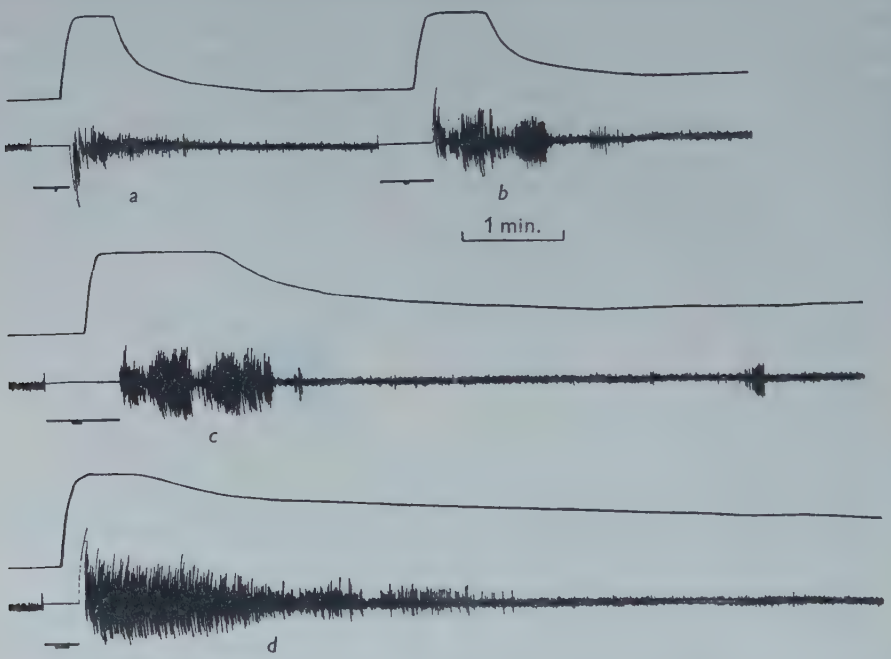

Fig. 10. The mechanical (upper trace) and electrical (lower trace) responses of the A.B.R.M. to increasing duration of continuous direct current. Stimulation for approximately: *a*, 2 sec.; *b*, 4 sec.; *c*, 6 sec.; *d*, 10 sec.

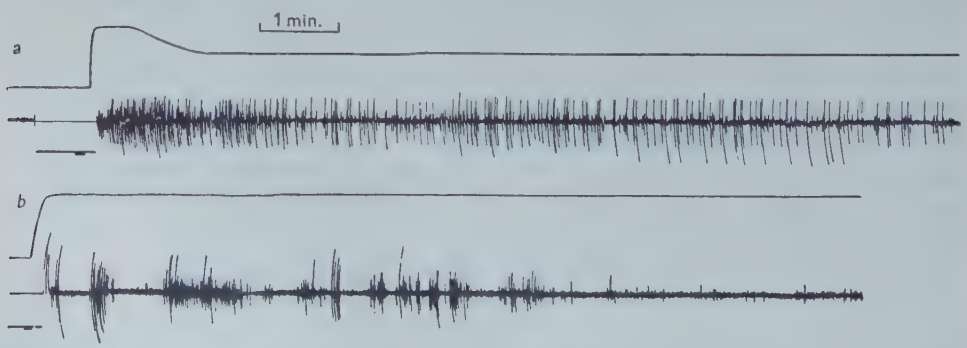

Fig. 11. Prolonged tonic contractions associated with continued electrical activity following d.c. stimulation. Upper trace tension, lower trace electrical activity. Note the variety of electrical activity recorded.

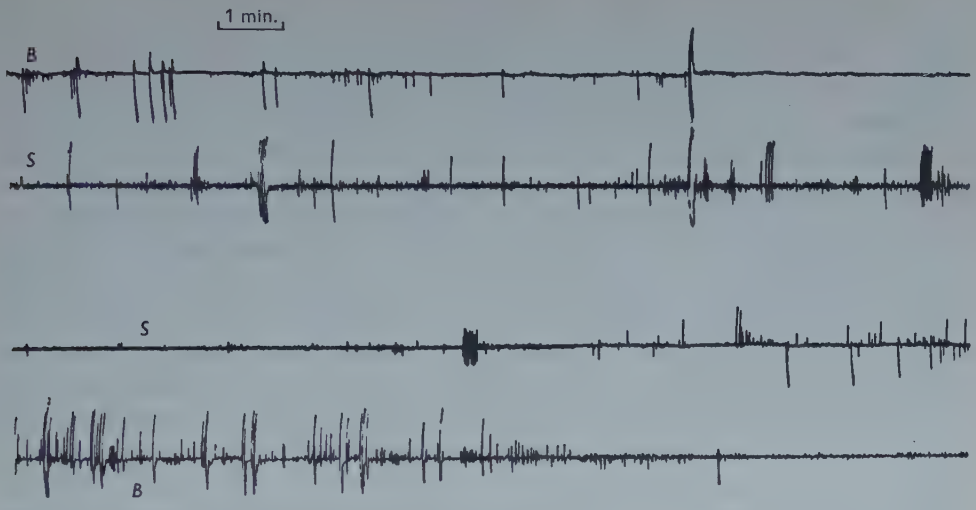

Fig. 12. Spontaneous electrical activity in the isolated A.B.R.M. recorded simultaneously during tonic contraction from regions near the byssus end (*B*)and the shell end (*S*). Note the localized nature of the activity.

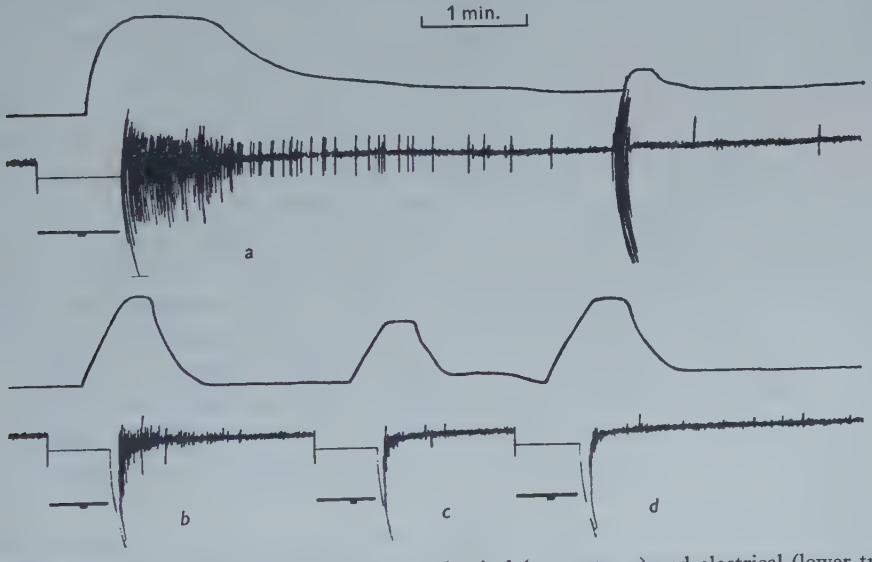

Fig. 13. The action of 5-H.T. (10^{-6}) on the mechanical (upper trace) and electrical (lower trace) responses of the A.B.R.M. to similar d.c. stimulations. *a*, before treatment; *b*, *c* and *d*, a few minutes after treatment.

DISCUSSION

Before discussing the significance of the present results it is necessary to consider observations made by Fletcher (1937) because on these are based most of the recent speculations which attempt to account for the behaviour of smooth lamellibranch muscles in terms of the 'catch' mechanism hypothesis. The potentials recorded by Fletcher had a duration up to fifty times longer than those observed in the present

work. More important, however, only a single such potential could be detected by Fletcher in response to a direct current applied to the isolated muscle, whereas our experiments show that prolonged electrical activity invariably follows such a stimulus. These contradictions can be resolved by comparing Fletcher's figures for the maximum gain and fastest response time of his recording devices with the corresponding figures for our own apparatus: it would appear that Fletcher's results were due to the relative insensitivity of his amplifier and recording apparatus.

With this difficulty out of the way, a reasonably clear account can now be given of the functioning of the *Mytilus* A.B.R.M. It is evident from the irregularity of much of the electrical activity and the variable magnitude of the spikes that the innervation of the muscle must be complex. Bowden & Lowy (1955) have recently demonstrated the presence of ganglion cells in the muscle. Furthermore, the different responses obtained from different regions show that there is no uniformity in the innervation of the different parts of the muscle.

As the electromyogram shows, the muscle works by continuous excitation of small groups of fibres, the number of such units in action at any time determining the level of tonus. Changes in the tonus level may be brought about by activation of motor or inhibitory axons via nerve cells located in the pedal ganglia. Phasic contraction could be due to transient stimulation of a large number of muscle units. The observation that the capacity of the muscle for tonus can be abolished, while it continues to give phasic responses, suggests the existence of two independent excitatory systems. It has not yet been possible to show whether the spontaneous activity of the system responsible for tonic contraction is neurogenic or myogenic. At all events, nervous pathways almost certainly serve for co-ordination of the numerous small muscle units during phasic contraction.

On the basis of the above considerations the effects produced by a.c. and d.c. stimulation can be interpreted as follows. Pulses and a.c. would usually bring about only a transitory increase in the rate of firing of the spontaneously active nerve (or muscle) elements. But a d.c. stimulus very often appears to lead to their persistent increased activity, thus giving rise to a prolonged tonic contraction. The d.c. could bring about ionic shifts which might influence spontaneous activity owing to a change in membrane potentials of the automatically-discharging elements.

Activation of 'inhibitory' axons reduces electrical activity in the isolated muscle, even when the level of activity is very high, as for instance following application of acetylcholine or d.c. stimulation. Similar results were obtained by stimulating the inhibitory nerves leading to the posterior adductor of *Mytilus* (Lowy, 1953). It may well be that activation of the inhibitory axons releases a substance which reduces the rate of firing of the spontaneously occurring activity.

There is one puzzling phenomenon which still has to be accounted for, namely, the observation of electrical activity during relaxation of the denervated A.B.R.M. (cf. Fig. 10*b*) and of the intact *Mytilus* adductor (Lowy, 1953). One of us (J.L.) has previously suggested that a process analogous to the β-inhibition, described by Marmont & Wiersma (1938) for crustacean muscle, could be responsible for producing this effect. However, in view of the present findings, an alternative explana-

tion is possible. It is obvious that, as the potential recorded is a measure of the electrical unbalance between the recording points, a small single muscle unit firing in a suitable position could produce a greater potential difference than a very large number firing synchronously—if the electrodes chanced to record from regions where similar events were taking place. Now because electrical activity is so widely scattered the observation of large muscle potentials during relaxation (even when few can be observed during contraction) is not really surprising: these spikes probably represent activity in only a few small units, which serves to slow down the rate of relaxation. The almost complete absence of electrical activity during really fast relaxation of intact muscles and on stimulation of inhibitory nerves (see also Lowy, 1953) constitutes good evidence in support of this explanation. The suggestion that electrical activity during relaxation indicates the operation of an active process which switches off the 'catch mechanism' (Barnes, 1955) is not likely to be correct.

The picture of the *Mytilus* A.B.R.M. which now emerges is that of a muscle controlled in many respects like vertebrate visceral smooth muscles: there is a built-in tonic system capable of automatic firing whose activity is normally regulated by motor and inhibitory axons from the central nervous system. The present experiments have shown how these two types of nerve can be brought into action by suitable electrical stimulation to produce muscular responses similar in every way to those observed in intact animals.

SUMMARY

1. The mechanism of contraction and relaxation in the anterior byssus retractor muscle (A.B.R.M.) of the lamellibranch mollusc *Mytilus edulis* has been studied with refined stimulating and recording techniques. Two distinct types of response are present: phasic and tonic. The former can be evoked by all kinds of electrical stimulation, but the latter is most readily elicited by continuous direct current.

2. Serotonin (5-hydroxytryptamine) rapidly abolishes the tonic response, whilst leaving the phasic response practically unaffected for many hours. On electrical stimulation, a serotonin-treated muscle may show spontaneous electrical and mechanical activity, usually of a rhythmical nature. As the muscle is unable to destroy this drug the effects produced by it are likely to be of pharmacological interest only.

3. Electrical activity in the form of irregular volleys of muscle action potentials can be recorded from the intact A.B.R.M. and even after the nerves to the muscle have been cut. In such (externally) denervated preparations the spontaneous electrical activity is almost unaffected by a.c. stimulation but greatly increased by d.c. stimulation which evokes prolonged tonic contraction.

4. Stimulation of inhibitory nerves within the muscle quickly terminates a state of tonic contraction and at the same time reduces the electrical activity considerably.

5. An explanation is offered for the occurrence of electrical activity during relaxation of lamellibranch smooth muscles. The normal functioning of the A.B.R.M.

is interpreted, and strong support is given to the tetanus hypothesis of tonic contraction.

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PROTEOLYTIC ACTIVITY OF THE MIDGUT IN RELATION TO FEEDING IN THE BEETLES *TENEBRIO MOLITOR* L. AND *DYTISCUS MARGINALIS* L.

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Where quantitative methods have been applied to the study of insect digestion, enzyme activity has generally been shown to increase after feeding, often with change in location of maximum enzyme concentration (Schlöttke, 1937*a–c*; Duspiva, 1939; Day & Powning, 1949; Fisk & Shambaugh, 1952). The most marked responses described by these authors occur in carnivorous and blood-sucking insects, and to some extent this supports the view put forward on histological grounds by Haseman (1910), that discontinuous secretion is associated with the carnivorous habit of taking discrete meals.

The work described here indicates the extent to which food regulates digestive secretion in *Tenebrio molitor* L. and *Dytiscus marginalis* L., two beetles of widely different feeding habit. *Tenebrio* passes its entire life cycle in its food (flour and like substances) and feeding appears superficially continuous during its larval stages, whereas *Dytiscus* is an aquatic carnivore whose meals depend on the fortunes of predation. Duspiva (1939) found protease discharge in *Dytiscus* to depend on taking food; this relationship is briefly re-examined for comparison with the situation in *Tenebrio*.

Hitherto the possibility of an endogenous rhythm in secretion related to age or development has been neglected, although such periodicities have been suggested on histological grounds in various beetles (Wigglesworth, 1953). As such a rhythm might mask effects due to feeding, secretory changes connected with development were examined in *Tenebrio* before testing the effect of food.

METHODS

(*a*) *General procedure*

Enzyme concentration in insect digestive tissues has usually been measured on extracts prepared from groups of similarly treated insects assumed to be physiologically homogenous. By recording enzyme values for individual insects, it was hoped that variation within experimental groups would be more readily appreciated.

Preliminary work with *Tenebrio* showed the enzyme content of the midgut to depend on age, stage of development and feeding history. Within any group of insects defined by these factors the range of enzyme values was wide, necessitating the use of large numbers of individuals before differences between groups became apparent. As the techniques involved precluded large numbers of concurrent

determinations, data were accumulated over a period of months, using sets of six to twelve insects from different experimental groups at a time. When further replication failed to alter the mean value of a group appreciably, it was considered adequately sampled. The definition of experimental groups is discussed later.

Examination of *Dytiscus* was limited to changes in secretion associated with feeding. These were sufficiently pronounced to be apparent from a comparison of individual beetles.

(*b*) *Preparation of standard enzyme extracts*

Similar techniques were followed in the preparation of gut extracts for both species, and in the subsequent evaluation of their protease activity.

Whole midguts were dissected from live *Tenebrio*, freed from adhering fat-body, and weighed on a torsion balance. For the measurement of tissue enzyme the midgut was slit open and washed free of contents in insect saline; after drying for a few seconds on filter-paper the washed tissue was weighed. The whole midgut or washed tissue was ground with a small quantity of kieselguhr in a thick-walled ignition tube, and 2 ml. of phosphate-buffered glycerin extraction fluid (Linderström-Lang & Duspiva, 1936) added with thorough mixing. After standing, with periodic agitation, for 1 hr., the tube was centrifuged at 3000 r.p.m. for 15 min., when the kieselguhr and tissue solids formed a compact pad from which the clear extract was easily decanted.

Separate extracts of the crop contents, midgut contents, and midgut tissue were prepared from each *Dytiscus*. Crop and midgut were dissected from the live beetle and removed to a watch-glass, where the former was transected at the cardiac valve, slit open, and its contents washed out in 2 ml. of extraction fluid. After thorough mixing this was removed to an ignition tube for centrifugation. The midgut contents were likewise dealt with. The washed midgut tissue was dried, weighed, and extracted as described for *Tenebrio*.

(*c*) *Protease determination*

The titimetric method of Day & Powning (1949) was followed with slight modification. A gelatin substrate was mixed in bulk, using 1 part of M/15 phosphate buffer of pH 8·0 to 2 parts of 6% bacteriological gelatin. 10 ml. of toluene were added to each litre of substrate mixture which could then be stored in a refrigerator without deterioration. For each experimental run 7·5 ml. of this substrate were mixed with 0·5 ml. of enzyme extract and incubated at 39° C. for 18 hr. 7·5 ml. of substrate plus 0·5 ml. of extraction fluid were used for blank runs. A drop of toluene was added to each run before incubation to prevent bacterial action. After incubation 0·5 ml. samples were titrated with N/40 alcoholic (90%) KOH as described by Day & Powning. The difference between titres for experimental and blank runs gave a measure of the protease activity of the extract. Transformation to arbitrary comparative units was effected by reference to a dilution curve prepared by plotting the titres for serial dilutions from a concentrated extract. Curves for both larval and adult *Tenebrio* extracts were similar, and one was selected for deriving all *Tenebrio* protease values. A separate dilution curve was prepared in a similar manner for use with *Dytiscus*.

(*d*) *Observations on the development of* Tenebrio *and their application in defining experimental groups*

A stock culture of *T. molitor* L. was obtained from the Imperial College insectary at Silwood Park, Berks, and maintained at a constant temperature of $27 \pm \frac{1}{2}°$ C. and a R.H. of $50 \pm 15\%$. The insects lived in a mixture of wholemeal flour and middlings in 7 lb. glass jars containing up to 500 larvae per jar. Individuals at appropriate stages of development were confined singly in $3 \times 1\frac{1}{2}$ in. glass specimen tubes, where their pre-experimental history could be accurately noted and adjusted. Thus isolated, times of moult, pupation and emergence were known, and growth curves were obtained by periodic weighing.

Classification into experimental groups was based on age in relation to a moult or emergence, and the occurrence or non-occurrence of feeding. Feeding was presumed to have occurred during any specified period if a weight increment was recorded. It could not be assumed that weight loss implied non-feeding, as this might result if defaecation exceeded food ingestion. Those designated 'unfed' were therefore deprived of food from moult or emergence. As the midgut is largely emptied at these times, the effect of variable storage of food was avoided.

In some larvae removed from food at moult, especially those in a late instar, further moulting could occur (Buxton, 1930; Leclercq, 1949). Thus it would have been possible for those classified as 'unfed' to be in a premoult or prepupal condition. However, a study of larval growth curves showed this to be unlikely in larvae less than 90 mg. in weight. If these had moulted once after removal from food, a state of starvation uncomplicated by imminent metamorphosis was reasonably assured.

From growth-curve data it was possible to determine approximately when a moult or pupation was about to occur in larvae developing on a normal diet. Weight increases regularly during the early part of each instar; feeding ceases shortly before a moult, which is preceded by a period of 5 to 6 days during which weight is lost (Murray, 1956). Dissection of the midgut at this time revealed greatly reduced contents. As freshly moulted larvae always had a small residue in the posterior part of the gas-distended midgut, it appears that complete evacuation only occurs before pupation. In prepupae, which may be recognized by their characteristic appearance (Stallwaag-Kittler, 1954), the midgut is completely empty.

Using the foregoing information it was possible to relate the age of any insect approximately to a moult, pupation, or emergence, and to classify it as 'fed' or 'unfed' in the sense defined above.

VARIATION IN MIDGUT PROTEASE IN ADULT *TENEBRIO*

The midgut protease of unfed adults was measured at various times after emergence. Separate insects were used to provide data for the total midgut (midgut tissue plus contents) and midgut tissue only. Protease values for the total midgut are recorded in Table 1, together with the means for each age group, and those for tissue only in Table 2.

The data of Table 1 were examined after separation into male and female sets, and group means for each sex are included. Though indicating slightly greater enzyme content in the female, the similarity between the sexes was considered sufficient to justify combining all data.

Table 1. *Total midgut protease in adult* Tenebrio *isolated without food from emergence*

Days after emergence ...	1	2	3	4	5	6	9	14	21
Individual protease values	2	7	11	25	30	41	19	5	11
	1	3	10	14	43	14	23	14	13
	1	6	6	36	40	50	27	19	6
	1	3	7	23	38	37	6	21	18
	0	1	6	31	34	38	34	20	16
	0	1	2	40	29	44	23	9	10
	0	0	8	20	34	39	23	20	4
	0	1	6	19	28	26	23	5	25
	1	3	8	19	43	25	11	17	3
	—	3	5	23	20	23	32	19	7
	—	—	9	—	—	—	46	30	—
	—	—	1	—	—	—	23	13	—
	—	—	—	—	—	—	41	15	—
	—	—	—	—	—	—	34	15	—
	—	—	—	—	—	—	22	18	—
	—	—	—	—	—	—	27	26	—
	—	—	—	—	—	—	—	17	—
Means (both sexes)	1	3	7	25	34	34	26	17	12
Means (males only)	1	3	6	20	33	31	25	13	15
Means (females only)	1	3	7	28	35	36	27	20	10

Table 2. *Midgut tissue protease in adult* Tenebrio

	Unfed from emergence								Flour taken within 24 hr. of dissection	
Days after emergence...	1	2	3	4	5	6	9	14	9	14
Individual protease values	0·0	0·0	1·5	3·0	1·0	4·5	3·0	1·5	2·0	3·5
	0·0	0·0	0·5	1·5	2·0	12·0	2·0	0·0	4·0	3·5
	0·0	1·0	1·0	4·0	1·0	2·0	0·0	0·0	2·0	2·0
	0·0	0·0	3·0	4·5	4·0	3·0	0·0	0·0	—	1·5
	—	1·0	1·0	12·5	2·5	5·5	1·0	0·5	—	—
	—	2·0	4·0	9·5	3·0	2·5	0·5	0·5	—	—
	—	—	2·5	1·0	2·5	2·0	0·0	0·0	—	—
	—	—	0·0	1·5	2·5	4·0	0·0	0·0	—	—
	—	—	1·5	11·0	—	—	1·5	0·5	—	—
	—	—	0·0	—	—	—	1·5	—	—	—
	—	—	—	—	—	—	2·0	—	—	—
Means	0·0	0·5	1·5	5·5	2·5	4·5	1·0	0·5	2·5	2·5

To assess the effect of feeding, unfed adults were allowed access to wholemeal flour, damp cellulose powder or water at various times after emergence, and periodic weighing so arranged that feeding was known to occur during particular periods preceding dissection. Values for total midgut protease of adults thus fed are listed in Table 3. Data for tissue protease are included in Table 2.

Table 3. *Total midgut protease in adult* Tenebrio *allowed to feed after various periods of starvation from emergence*

	Food withheld 13 days after emergence: ingestion occurred during 14th day					
	Flour				Cellulose powder	Water
Days after emergence ...	6	9	14	17	14	14
Individual protease values	32	41	42	32	54	48
	26	21	48	32	24	31
	45	39	41	—	24	34
	35	31	53	—	18	22
	32	58	48	—	12	17
	—	—	33	—	14	20
	—	—	23	—	28*	42*
	—	—	28	—	42*	45*
	—	—	19*	—	42*	45*
	—	—	22*	—	76*	22*
	—	—	—	—	16*	24*
	—	—	—	—	37*	29*
	—	—	—	—	15*	—
Means	34	38	36	32	31	32

* Ingestion occurred within 4 hr. of dissection.

In Fig. 1 group means for tissue protease and total protease of unfed adults are plotted against time after emergence. The two curves fitted visually to the points so obtained are taken to represent the fluctuation of protease in the midgut as a whole, and in the midgut epithelium. At emergence proteolytic enzymes are lacking. They build up from the 1st day after emergence until total midgut protease reaches a maximum between the 5th and 6th post-emergence days, with the greatest rate of increase occurring on the 3rd and 4th days. Thereafter, it gradually decreases on continued starvation until death ensues. Tissue protease, which becomes comparatively high between the 4th and 6th days, falls to a negligible residual value by the 9th day, and remains so until death.

Comparison of the means for total protease in fed adults (Table 3) with those unfed of the same age (Table 1) indicates the effect of feeding. Between the 5th and 6th days after emergence, when in unfed adults the spontaneous increase is maximal, the amount of protease accumulated is unchanged. At later times, when in the absence of feeding total protease is reduced, the ingestion of flour causes an increase to a value approximating that attained initially. Similar events follow the ingestion of damp cellulose or water, and are apparent within 4 hr. In adults fed flour at these later times tissue protease rises from the residual starvation level to values comparable with those reached spontaneously after emergence (Table 2).

These results show that the secretion of protease is stimulated by endogenous events at emergence, and after the ingestion of food (or nutritionally valueless material) in the mature adult. From a comparison of the values for tissue protease and total protease it is clear that the latter is largely a measure of discharged enzyme in the midgut lumen, epithelial accumulation being negligible. This being so, tissue

protease may reasonably be considered an index of the rate of synthesis of enzyme in the secretory cells. As large increases in total (i.e. discharged) enzyme occur when tissue enzyme is comparatively high, it appears that synthesis and discharge form an integrated process.

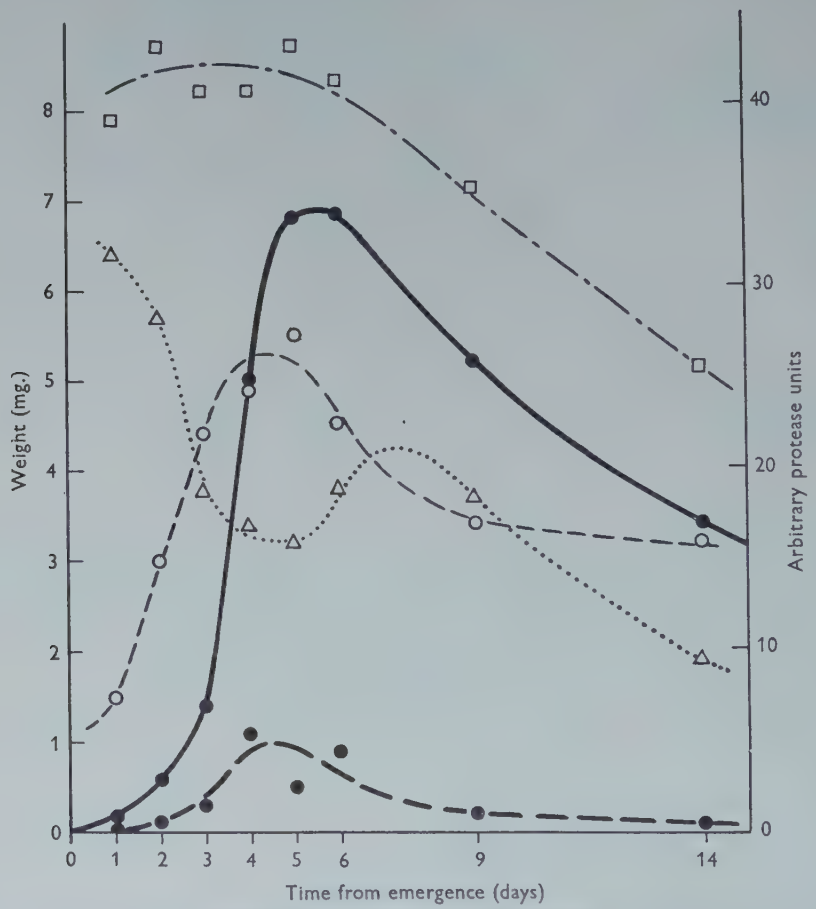

Fig. 1. Changes in midgut weight and protease in adult *Tenebrio*. —●—●—●—, total midgut protease; —●—●—●—, midgut tissue protease; —○—○—○—, weight of midgut tissue; —□—·—□—·—□—, weight of total midgut; ···△···△···△···, weight of midgut contents.

VARIATION OF MIDGUT WEIGHT IN ADULT *TENEBRIO*

When tissue protease was measured weights of total midgut and washed midgut tissue were recorded. These are listed in Table 4 and group means plotted against time after emergence in Fig. 1, together with the values obtained by subtracting tissue weight means from corresponding total weight means. The curves fitted visually to these points represent the fluctuation of tissue weight, total weight and weight of midgut contents with time, and may be compared with the curves showing variation in tissue and total protease.

Corresponding changes in the weight and protease of the tissue are evident.

Tissue weight increases as tissue protease rises from zero at emergence, and falls to a more or less stationary value as protease decreases to a low residual level; increase in weight marks the increase in tissue enzyme which follows feeding. The initial weight increment is unlikely to represent formed enzyme. This has been shown to accumulate mainly in the midgut contents, and no corresponding weight increase occurs for the total midgut. It follows that epithelial growth occurs during active secretion. This conclusion accords with the observation of accelerated mitosis in the midgut regenerative cells of *Tenebrio* during secretion (Day, 1949), and supports the view that epithelial regeneration is an important feature in the secretory process of some insects.

Table 4. *Weights of total midguts and midgut tissues in adult* Tenebrio

	Unfed from emergence								Flour taken during previous 24 hr.	
Days after emergence ...	1	2	3	4	5	6	9	14	9	14
Individual weights of midgut tissue (mg.)	1·3	2·6	3·2	5·0	6·6	3·4	3·2	4·2	4·5	3·8
	1·2	2·8	3·4	5·2	5·8	3·5	3·2	2·4	4·0	4·9
	1·6	3·6	3·6	3·9	4·7	4·2	2·4	3·5	5·0	2·8
	1·7	3·7	4·3	4·6	4·9	4·3	4·1	3·1	—	3·5
	—	2·3	3·5	4·2	4·7	5·6	2·4	3·5	—	—
	—	2·8	3·7	5·2	6·8	5·3	5·3	2·6	—	—
	—	—	3·5	5·2	5·4	3·1	3·0	1·8	—	—
	—	—	6·5	4·9	4·7	6·0	3·7	2·9	—	—
	—	—	5·4	4·9	—	—	3·5	5·0	—	—
	—	—	7·1	—	—	—	3·9	—	—	—
	—	—	—	—	—	—	2·8	—	—	—
Tissue weight means	1·5	3·0	4·4	4·8	5·5	4·5	3·4	3·2	4·5	3·8
Weights of individual total midguts (mg.)	8·0	6·1	6·0	6·6	10·8	8·1	5·1	9·1	8·8	8·5
	7·6	9·1	5·1	8·8	8·5	9·1	5·0	4·2	9·3	10·0
	8·2	8·8	7·7	8·3	6·8	8·6	4·4	4·7	8·2	7·6
	7·9	11·0	9·3	8·1	7·3	7·7	6·5	4·2	—	8·8
	—	9·0	7·9	5·3	8·7	8·1	6·5	5·0	—	—
	—	8·4	6·4	11·0	10·7	10·5	8·4	3·7	—	—
	—	—	8·8	8·5	8·1	6·5	5·1	3·2	—	—
	—	—	11·2	9·2	8·7	8·9	9·0	5·0	—	—
	—	—	9·9	8·7	—	—	7·6	8·6	—	—
	—	—	10·0	—	—	—	6·0	—	—	—
	—	—	—	—	—	—	7·6	—	—	—
Total weight means	7·9	8·7	8·2	8·2	8·7	8·3	7·1	5·1	8·8	8·7
Difference between means (weight of midgut contents)	6·4	5·7	3·8	3·4	3·2	3·8	3·7	1·9	4·3	4·9

As total midgut weight remains constant during tissue growth, midgut contents must diminish. At first sight growth appears to be at the expense of midgut contents, but these weight relationships would hold if tissue growth at the expense of body fluid were accompanied by a loss of contents from the midgut. The available data provide no means of distinguishing between these possibilities.

In the unfed adult total midgut weight falls steadily from the 6th to the 14th day. As tissue weight remains nearly constant after the 9th day this decrease must be due to the evacuation of midgut contents, and largely accounts for the decline in

total midgut protease over this period. From the 5th to 9th days tissue weight decreases more than total weight; it may be supposed that during this period gut contents are augmented from tissue material, perhaps by the disruption of senescent cells as secretion declines.

VARIATION IN MIDGUT PROTEASE IN THE *TENEBRIO* LARVA

On cursory examination secretory events in the larva proved similar in many respects to those of the adult, so fewer individuals were used for each experimental group In order to limit variation due to size, only larvae weighing between 75 and 90 mg. at the time of dissection were used; it was hoped that this might incidentally result in the majority being of the same instar. (Instar number is not easily determined in *Tenebrio*.) Furthermore, larvae of this weight were unlikely to be entering the physiologically complicated prepupal state. Protease values for the total midguts of larvae, grouped according to the criteria described earlier, are listed in Table 5.

Table 5. *Total midgut protease in* Tenebrio *larvae*

Time from moult (in days) ...	Without food from emergence											Premoulting larvae		Pre-pupae
	¼	½	1	2	3	4	5	6	7	14	42	−3	−2	
Individual protease values	1	12	21	19	26	16	15	23	42	23	11	16	4	0
	18	12	13	19	16	16	9	30	30	12	9	23	3	0
	13	14	11	11	10	16	12	18	11	26	13	16	—	—
	0	6	16	22	15	15	15	19	15	25	15	10	—	—
	14	18	14	17	22	16	23	17	20	29	17	—	—	—
	5	7	19	16	—	18	20	16	17	15	—	—	—	—
	—	10	—	14	—	27	17	26	—	12	—	—	—	—
	—	—	—	—	—	15	—	—	—	—	—	—	—	—
Means	9	11	16	17	18	17	16	21	23	20	13	16	4	0

Flour taken during the 24 hr. preceding dissection

Days after moult ...	Flour available from moult									Flour withheld for 21 days after moult		
	4	5	6	7	8	10	11	12	16	22	23	26
Individual protease values	32	43	37	42	28	30	40	27	27	35	44	33
	—	49	57	—	—	36	—	27	23	29	31	—

In contrast to the newly emerged adult, the larva has considerable protease immediately after moulting. Part of this may be ascribed to the carry-over of residual gut contents from the previous instar. Comparison with the build up of protease in the young adult suggests that secretion may commence before moulting is externally apparent. During the day after moulting protease rises to a stationary level; a further slight increase may occur between the 5th and 7th post-moult days, but the data are insufficient to decide this conclusively. Thereafter, total protease

decreases very gradually, contrasting in this respect with the adult, where enzyme activity in the midgut is halved after a week's starvation. The high level of protease conserved over long periods suggests both an efficient retention of gut contents (of importance to the larva in its ability to withstand months of starvation and desiccation) and continuous secretion at a low rate.

In larvae that have fed within the preceding 24 hr. protease values are higher than in those unfed from moult. This difference is very pronounced at the start of a feeding period, whether this follows normally from moulting, or after starvation. As feeding proceeds, the difference from the starvation level of enzyme decreases, and may indicate a diminished secretory response to feeding or an increase in the rate of evacuation of gut contents.

It may be noted that the second spontaneous increase in enzyme occurs 5 days after moulting, when secretion is maximal if food is available. This suggests that a tendency to continuous secretion has developed in association with regular feeding. If so, secretion may here be supposed an endogenously initiated function of the digestive epithelium subject to a limited regulation in response to stimuli arising during feeding.

Shortly before moulting the larva loses weight as feeding ceases, and the low enzyme values then recorded may be ascribed to the evacuation of most of the midgut contents at this time. Complete evacuation precedes pupation; in two prepupae examined the empty gut lacked proteolytic activity.

A systematic study of midgut tissue protease in the larva was not made. However, while working on enzyme distribution in the midgut epithelium, tissue protease was found to be of the same order of magnitude in larvae and adults. It may be concluded therefore that, as in the adults, little protease is retained in the epithelium.

PROTEASE SECRETION IN *DYTISCUS*

Adult *D. marginalis* L. were obtained from a dealer and isolated in 7 lb. glass jars containing about 2 in. of tap water at room temperature. (This varied between 15 and 20° C. during the course of the experiments.) On reception they were allowed to feed to repletion on whalemeat, removed to clean water devoid of ingestible material, and starved for 1 month. Preliminary work had shown that after this period the meat was completely digested and the crop empty. Beetles thus starved were offered meat, and, at various times after commencing to feed, were dissected for the measurement of gut protease. The data for one set of insects are listed in Table 6 and represented graphically in Fig. 2. Two other sets, though less detailed, gave essentially similar results.

Clearly, tissue protease is high during starvation and very low subsequent to feeding, whereas the converse applies to protease in the crop. Within 15 min. of feeding tissue enzyme is much reduced, and after an hour protease in the midgut tissue and lumen is virtually zero. Enzyme lost from the midgut appears in the crop; this organ contains over 90% of the total protease an hour after taking food. Protease recurs in the midgut tissue 3 hr. after feeding; by this time the synthesis

of additional enzyme must have started in the epithelial cells. Protease steadily increases in the crop and midgut contents during the 12 hr. after feeding. As midgut tissue remains low during this period, it may be inferred that the synthesis of fresh enzyme is accompanied by its continual discharge and translocation forward to the crop.

Table 6. *Protease in the crop and midgut of* Dytiscus

Sex	Wt. of beetle (g.)	Wt. of midgut tissue (mg.)	Time from commencement of feeding	Protease values			
				Crop contents	Midgut contents	Midgut tissue	Total
Female	1·7	11	15 min.	35	24	2	61
Female	1·8	17	1 hr.	54	4	0	58
Male	2·3	16	3 hr.	74	7	1	82
Female	1·9	29	12 hr.	*c.* 150	30	6	180
Female	2·0	27	24 hr.	45	6	4	55
Male	1·9	22	24 hr.	31	53	11	95
			Mean, 24 hr.	38	30	8	75
Female	1·8	32	2 days	54	3	8	65
Female	1·7	14	5 days	90	24	11	125
Female	1·7	31	2 weeks	0	46	36	79
Female	2·0	29	4 weeks	12	21	26	59
Male	1·6	14	4 weeks	1	20	13	34
			Mean, 4 weeks	7	21	20	47
Female	1·9	25	5 weeks	3	35	24	62
Female	1·9	17	7 weeks	0	4	7	11

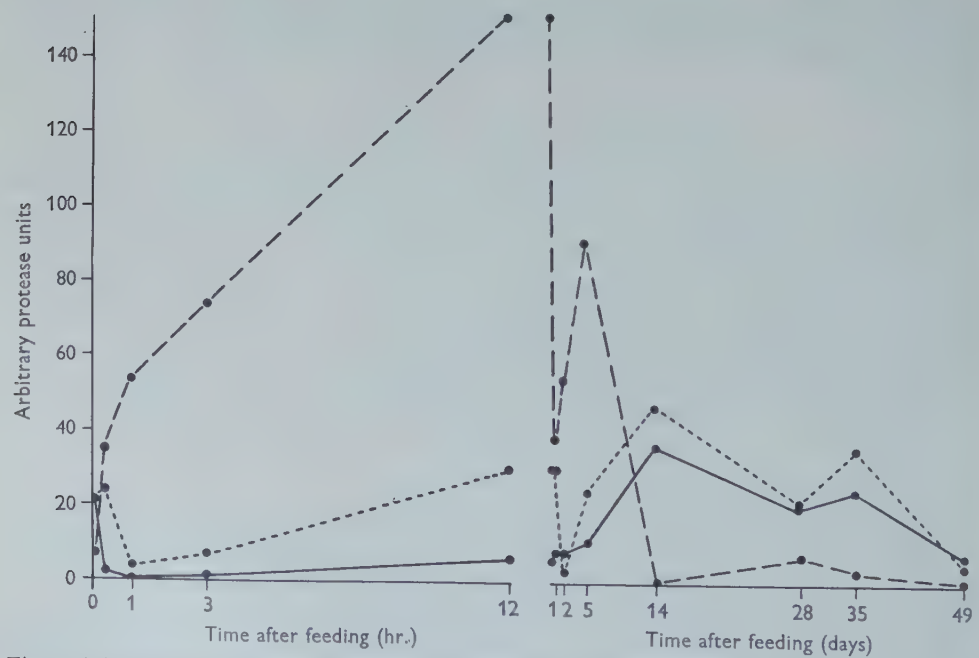

Fig. 2. Distribution of protease in the gut of *Dytiscus* during digestion and subsequent starvation. ————, midgut tissue; ------, midgut contents; — — — —, crop contents.

For some days after feeding tissue protease remains low. During this period the variable distribution of enzyme between crop and midgut lumen is best accounted for by the periodic evacuation of the products of digestion in the crop backwards through the midgut. By the second week tissue protease has built up to a high level, and thereafter it slowly decreases if starvation is prolonged. When tissue protease is maximal, crop protease is virtually zero, and it remains so on starvation; when this condition obtains the crop is found to be empty.

These results suggest that while the crop contains undigested material, continual synthesis and discharge of protease occur from the midgut epithelium. On complete evacuation of the crop discharge ceases, allowing the accumulation of enzyme in the epithelium. This view differs somewhat from that of Duspiva (1939), who on histological grounds considered the epithelial cells in a 'resting condition' a few hours after the initial discharge. Though the foregoing results might be accounted for in terms of a secretion cycle of a few hours' duration, histological examination always revealed the characteristic condition associated with enzyme discharge if the crop contained undigested contents. These discrepant observations may perhaps be due to differing conditions of feeding and amounts of food taken. It is clear, however, that with continued starvation discharge of enzyme ceases and allows epithelial reserves to be accumulated. This separation of the processes of synthesis and discharge contrasts markedly with the condition in *Tenebrio*.

DISCUSSION

Information on the development of digestive function in relation to the moulting cycle of insects is meagre. Where extensive reorganization of gut tissues is involved secretion might be expected to decrease or cease as in *Tenebrio*. Uvarov (1928) mentions similar changes in some lepidopterous pupae. Spontaneous secretion after moulting or emergence is readily understood in *Tenebrio* where food is normally always available. Enzyme activity increases after moulting in larvae of *Limnophilus* and *Bombyx* (Roques, 1909; Roeder, 1953), which likewise live near their food. The newly emerged female *Aëdes*, on the other hand, has negligible protease until blood has been taken (Fisk, 1950; Fisk & Shambaugh, 1952). In this mosquito the absence of enzyme preparatory to feeding may be of significance in relation to the sporadic occurrence of blood meals.

Many authors have been concerned to relate digestive secretion to feeding behaviour. Though quantitative studies have usually demonstrated increased enzyme activity after feeding, it has been argued that in insects which feed continuously, particularly in larvae with food always available, a mechanism for the stimulation of secretion at the time of feeding may not be required (Haseman, 1910; Schlöttke, 1937*b*; Pradhan, 1939). Particular interest therefore attaches to the stimulation of secretion by food in the *Tenebrio* larva, where, superficially, feeding seems continuous. Crowell (1943) considered most cases of reputed continuous feeding to be doubtful. Critical examination usually reveals that though the gut is kept full ingestion is intermittent, and this appears the case with *Tenebrio* larvae (Murray, 1956).

On the available information secretory situations are more profitably related to the amount of food normally maintained in the gut. On this point Day & Powning (1949) distinguish between 'effectively continuous' feeders which maintain a more or less full gut, and discontinuous feeders. In the former, continuous secretion subject to regulation in rate might be anticipated, as in the roaches they studied. Intermittent secretion is most likely where the gut may frequently be empty, as in predators dependent for meals on occasional prey.

Tenebrio and *Dytiscus* illustrate this distinction in feeding habit and have been shown to possess distinctive secretory mechanisms. Secretion in *Tenebrio* may be supposed a continuous process initiated at moult or emergence and accelerated after feeding. This seems especially likely in the larva, where it is difficult otherwise to account for the high level of protease maintained during protracted starvation. In *Dytiscus*, on the other hand, the independence of discharge from synthesis, by allowing the intra-epithelial accumulation of enzyme during starvation, results in secretion as a whole being markedly intermittent. The work of Fisk & Shambaugh (1952) on *Aëdes* supports the correlation of intermittent secretion with irregular feeding, though in detail the secretory mechanism involved is very different from that of *Dytiscus*. As a working hypothesis, the view that intermittent secretion and irregular feeding are associated has some use, but it is clear that wider study will show diversity in detail.

Thus far it has been tacitly understood that considerable information may be obtained from an examination of single enzymes by supposing them to indicate secretion of the digestive juice as a whole. Duspiva (1939) considered secretion in *Dytiscus* to occur as a single process, and the observation of concurrent increases in various midgut enzymes argues a similar situation in some Orthoptera (Schlöttke, 1937*b*, *c*; Day & Powning, 1949). A uniform digestive juice seems probable in *Tenebrio*. Although in preceding sections only proteolytic activity has been described, amylase values were determined for some extracts, and were high where protease was high. Further, the similar results obtained by feeding either flour, cellulose powder or water to adults suggest a general secretory response rather than the separate stimulation of individual enzymes. Differential secretion of protease and amylase in response to their appropriate substrates occurs in *Aëdes*, and may be related to the need for occasional blood meals for ovarial maturation in an insect whose main diet consists of plant juices (Shambaugh, 1954). In *Tenebrio* and other insects whose normal diet always requires their full complement of enzymes differential secretion would effect no improvement in digestive efficiency.

Of great interest in this connexion is the postulation of different modes of stimulation of the secretory cells. Shambaugh (1954) shows secretogogues to be active in *Aëdes*, whereas Day & Powning (1949) suggest that a hormone-like blood factor provides the immediate stimulus in *Tenebrio* and roaches. As substances lacking the chemical qualities of normal food are effective in stimulating secretion in *Tenebrio* adults, the response seems to be dependent on some mechanical factor involved in ingestion or feeding behaviour rather than a gustatory or secretogogue effect elicited by specific nutrients. This accords with the hypothesis of Day &

Powning. Further, as endogenously initiated secretion occurs at moult and emergence in the absence of food and therefore of secretogogues, its initiation may well be an integral part of the hormone-regulated events of metamorphosis.

SUMMARY

1. In *Tenebrio* secretion of protease occurs spontaneously after moult and adult emergence, and in response to feeding in the active larva and mature adult. Damp cellulose powder or water are effective in increasing secretion in the adult.

2. Since little enzyme is accumulated in the epithelial tissue when the total midgut enzyme is greatly increased, it is inferred that synthesis and discharge are interdependent. When synthesis (as indicated by comparatively high tissue enzyme) is accelerated, growth of the midgut epithelium occurs.

3. In starved *Dytiscus* protease is accumulated in the midgut tissue. Within one hour of feeding it is largely discharged into the crop. Protease recurs in the midgut tissue in a few hours, but remains low so long as the crop contains undigested material. When the crop is empty, discharge ceases and enzyme is again accumulated in the epithelium. Thus the process of discharge appears to be independent of synthesis.

4. The secretory mechanisms of *Tenebrio* and *Dytiscus* are discussed in relation to their feeding habits.

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REPRODUCTION AND GROWTH OF MICE OF THREE STRAINS, AFTER TRANSFER TO −3° C.

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'Wild' house mice (*Mus musculus* L.) breed in cold stores kept at about −10° C (Laurie, 1946), and laboratory mice of the same species can be bred at an environmental temperature of −3° C., given nesting material (Barnett & Manly, 1954; Barnett, 1956). The response of mice to low temperatures varies with different inbred strains. The differences are reflected in mortality (especially of young), growth, numbers of young born and weaned, and in internal, especially endocrine, changes. All these, and other aspects of the maintenance of the lives of mice at low temperatures, are to be discussed in future communications.

This paper deals with the effect of low temperature on the reproduction of mice of three strains. The experimental mice were transferred as young adults from a 'normal' temperature of about 21° C. to 10° C., or to −3° C., and mated at the same time. The study of the reproductive performance and growth of these mice provides a quantitative expression of differences between the strains.

MICE AND METHODS

The mice were of the following strains: (i) A (from Imperial Cancer Research Fund, Mill Hill, London); (ii) C57BL (also from Mill Hill); (iii) GFF (from Glaxo Laboratories, Greenford, Middlesex). All three strains have a long history of rigorous brother-sister mating.

Three constant-temperature rooms were used. The conditions in the two main rooms, kept at −3 and 21° C. respectively, and cages, food and nest material, are described in a previous paper (Barnett, 1956). The third room, kept at 10° C., was, unlike the others, not designed for the research on mice, and had a fan which maintained a continuous air circulation. It therefore differed in air movement, and in noise, as well as in temperature, from the other rooms.

Breeding procedure was as follows. All the mice to be mated were reared at 21° C. At 3 weeks they were separated from their parents, and at 5 weeks the sexes were separated. At 7 weeks all experimental mice were transferred to the room kept at 10° C., while the control mice, also not yet mated, remained at 21° C. At 8 weeks the mice destined for −3° C. were transferred to that temperature, and all mice were mated. All pairs were litter-mates.

All the mice mated at −3° C. had litter-mate counterparts at 21° C., and all those mated at 10° C. had litter-mates at both the other temperatures.

Body weights of males, and reproduction, were recorded for 20 weeks, that is, up to the age of 28 weeks. All litters born in this period were included in the record, even if they were weaned only after the end of the period. Weaning weights, at 3 weeks, of litters of all pairs were recorded. A few of the litters were not weighed, and so the total numbers of young were slightly higher than the numbers of those whose weights are analysed.

RESULTS

Mice of all three strains survived the transfer to the cold room. Only in the C57 strain was there mortality attributable to the cold (Table 1); in this strain the mortality at $-3°$ C. is highly significant.

Table 1. *Mortality and reproduction up to age 28 weeks*

	A			C57BL			GFF		
	21° C.	10° C.	−3° C.	21° C.	10° C.	−3° C.	21° C.	10° C.	−3° C.
No. of pairs	12	4	12	14	8	14	13	3	13
Adult mortality ♂	0	0	0	0	0	4	0	0	0
Adult mortality ♀	0	0	0	—	1	1	0	0	1
No. of barren pairs	0	0	0	1	0	9	0	0	1
No. of pairs weaning no young	0	0	2	1	0	11	2	1	9
Mean litters per pair	4·6	3·5	3·6	3·2	2·8	1·0	3·3	2·3	1·5
Mean young weaned per pair	18·75	16·25	12·75	16·0	15·4	1·4	9·8	10·0	0·9
Mean young born per litter	5·0 ± 0·27	6·3 ± 0·64	5·2 ± 0·58	7·2 ± 0·58	7·2 ± 0·54	4·5 ± 0·48	3·9 ± 0·29	4·3 ± 0·93	3·4 ± 0·29
Mean young weaned per litter	4·1 ± 0·31	4·6 ± 0·74	3·5 ± 0·37	5·0 ± 0·37	5·6 ± 0·69	1·4 ± 0·53	3·0 ± 0·36	3·4 ± 1·23	0·6 ± 0·32

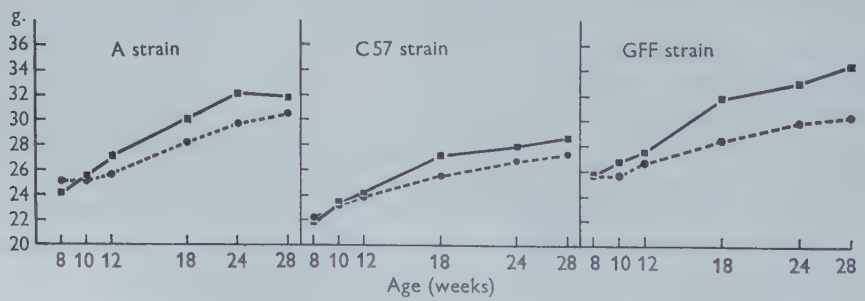

Fig. 1. Group means of weights of adult male mice. Continuous line: at 21° C. Broken line: at −3° C.

The group means of the weights of the males at six ages are shown in Fig. 1. At 21° C. the C57 mice are a smaller stock than the others at all ages; the GFF mice maintain a relatively high growth rate for longer than the A, and so are ultimately heavier in the warm. In the cold, total body growth is reduced in all three strains. The reduction is greatest in the GFF mice, and these, at the low temperature, no more than keep pace with the A mice. In both A and GFF mice

there was a marked check in growth during the first 2 weeks in the cold. Changes in relative growth will be reported separately.

Reproductive performance is summarized in Table 1. The figures for 21 and $-3°$ C. are the important ones, since the number of pairs at 10° C. is too small for full significance.

The number of litters born per pair was reduced at $-3°$ C. for all strains, but especially for C57 and GFF. There was no indication, from the weight records, of resorption of litters *in utero* in any of the mice at 21° C. At $-3°$ C. weight fluctuations suggest that this occurred in one A female, one C57 female and three GFF females. Complete failure to reproduce at $-3°$ C. occurred in none of the A pairs; but 64% of the C57 matings and 38% of the GFF matings were barren. The ability of the C57 mice to breed continuously in the cold (Barnett & Manly, 1954) therefore depends on the performance of a minority of successful pairs.

There is also, in the C57 and GFF strains, a heavy mortality in the cold during the first 3 weeks of life of the young, shown in the difference between the number of young born, and the number of young weaned, per litter. Data on conditions in the nest, relevant to this loss, are given in a previous paper (Barnett, 1956). The effect of cold on the number of young born *per litter* was slight; indeed, in the A mice no effect was evident.

Table 2. *Weights of young at 3 weeks*

	21° C.	10° C.	$-3°$ C.
A ♂ No.	111	34	70
Mean wt., g.	10·07 ± 0·20	9·41 ± 0·31	8·79 ± 0·19
♀ No.	110	31	87
Mean wt., g.	9·91 ± 0·20	9·10 ± 0·27	8·74 ± 0·20
C57BL ♂ No.	101	37	6
Mean wt., g.	7·88 ± 0·14	8·14 ± 0·27	6·08 ± 0·65
♀ No.	82	33	6
Mean wt., g.	7·60 ± 0·18	7·91 ± 0·29	6·83 ± 0·38
GFF ♂ No.	67	17	4
Mean wt., g.	9·96 ± 0·24	8·32 ± 0·33	9·50 ± 1·24
♀ No.	56	8	8
Mean wt., g.	9·79 ± 0·36	9·75 ± 0·50	10·31 ± 0·49

The weights of the young were significantly reduced at $-3°$ C. in the A and C57 strains, but not in the GFF (Table 2). We have evidence, to be published separately, that (as would be expected) resistance to cold at weaning is positively correlated with relatively high body weight. The weanling mice from the cold room, of which the weights are given in the right-hand column of Table 2, probably represent a group from which the lighter members have been eliminated during the 3 weeks in the nest.

The most important criterion of fertility is the number of young weaned per pair. In this respect there is little difference between the A and C57 strains at 20° C., but a marked difference at $-3°$ C. The A mice were notably successful at

the low temperature, showing a reduction of only 32% in the production of viable young; while the C57 mice showed a reduction of 91%. The performance of the GFF mice in the cold was even worse than that of the C57 mice, but comparison with their performance in the warm shows that the reduction in fertility was the same as that of the C57 mice, namely, 91%.

The results of breeding at 10° C. indicate that, in the conditions of the experiment, living at this temperature does not constitute a severe handicap for any of the three strains. This conclusion is further supported by the results of continued breeding, which will be published separately.

DISCUSSION

There is little published information on the effects of low environmental temperature on *reproduction* in mammals. Lee (1926) found that the oestrous cycle in albino rats was lengthened by exposure to outdoor winter temperatures. Parkes & Brambell (1928) found that transfer of laboratory mice from an environment at about 18° C. to one at about 0° C. led to a disturbance of the oestrous cycle, but this was only temporary: the length of dioestrus rose from a mean of about 4 days to one of about 11 days, but after the first week the normal cycle was restored. The mice were also mated at 0° C., and were found to be 'normally fertile'; they were however removed from the low temperature once pregnancy had been established. These observations are in conformity with our observations on A mice: in particular, we have found that the oestrous cycle in these mice, once they are established in the cold room, is normal (unpublished).

Laurie (1946) examined a large number of mice trapped in cold stores, and compared them with mice trapped in other habitats. Her estimated number of litters per female per year for mice in ricks (the most favourable habitat) was 10·2; the corresponding figure for our A mice at 21° C. is 11·5, assuming that breeding would continue at the same rate for 52 weeks as for 20. Laurie's figure for cold store mice was 6·7, ours for A mice at −3° C. is 9·0. The cold store mice, unlike our inbred mice at −3° C., were heavier than those in other habitats. However, the cold store mice studied by Laurie, besides being genetically heterogeneous, were living on a purely meat diet, at an environmental temperature well below that of our cold room. The comparison can therefore not be pursued very far.

A more potentially fruitful comparison is that between the three inbred strains used in the present experiments. The differences they show, in response to cold, while not surprising, suggest that a comparative approach to the study of reproduction in a cold environment is possible. Young (1953) has described an experiment in which genotype-environment interaction was studied in three inbred mouse strains; he points out that, given the use of several strains and environments, nature-nurture interactions of interest can be detected. In general, a decline in reproductive capacity, measured by the number of live 3-week young produced per pair, could be due to a great variety of effects. Sawin & Curran (1952) have studied reproduction in a number of rabbit strains at ordinary temperatures. They describe differences in maternal behaviour, in milk production and in growth rate,

as well as in production of young. In our mice the cold undoubtedly influenced the separate physiological processes involved in reproduction to varying degrees in the different strains. Not only were the A mice less affected at all points than the other strains, but, for example, the GFF mice showed a smaller reduction than the C57 mice in the young born per litter in the cold; on the other hand, the loss of young in the nest was higher for GFF than for C57 mice.

The present study deals only with the reproductive performance of mice born and reared in a warm environment. The full record of A and C57 stocks maintained for several generations in the cold will be published separately. It can be said here, however, that such stocks do not recover their lost fertility in later generations in the cold: in other words, the reproduction of pairs introduced into the cold as young adults is quite similar to that of pairs reared at −3° C. The later generations of C57 mice are of course descended from those pairs (as we have seen, a minority) which reproduce well in the cold. There may be involved, therefore, selection of mice genetically different from the rest, in cold resistance or reproductive capacity. This is possible, even though the mice are rigorously inbred, since it is unlikely that complete genetical uniformity exists in these strains. The question of selection for cold resistance will receive attention in later work.

SUMMARY

Mice of strains A, C57BL and GFF were mated at environmental temperatures of 21 and −3° C., with a few pairs also at 10° C. All pairs had nesting material.

Growth was reduced at −3° C. in all strains. Weights of A and C57, but not GFF, young on weaning at 3 weeks were reduced at −3° C.

The number of litters per pair, and of young weaned per pair, was reduced at −3° C. for all strains, but especially C57 and GFF. The fertility of the GFF strain at 21° C. is lower than that of the other strains: consequently, the GFF mice did very badly in the cold.

Reproductive performance at 10° C. was similar to that at 21° C. for all strains.

We are grateful to the Nuffield Foundation for grants which made this work possible; to Dr D. S. Falconer for discussing the analysis of the results; and to Margaret Doyle and Elizabeth MacMurray for technical assistance.

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STUDIES ON THE RESPIRATION OF SEA-URCHIN SPERMATOZOA

II. THE CYTOCHROME OXIDASE ACTIVITY IN RELATION TO THE DILUTION EFFECT

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In the previous paper (Mohri, 1956), it was shown that in sea-urchin spermatozoa, the outburst of respiration which occurs on dilution of a sperm suspension can be suppressed by sodium azide. However, the low respiration which follows the outburst is not affected by this poison, although both are inhibited by cyanide.

Similar observations have been made on other materials as regards azide action. As far as the eggs of sea-urchins are concerned, the respiration of unfertilized eggs was found to be cyanide-sensitive but azide-insensitive, while the increased respiration after fertilization was sensitive to both inhibitors (Fischer, Henry & Low, 1944; Robbie, 1946). Runnström (1932) and Örström (1932) found that the addition of sufficient amounts of dimethyl-*p*-phenylenediamine (dpphd) caused similar increases in the O_2 uptake of unfertilized and fertilized eggs. This was considered to show that, although the capacity of the echinoderm oxidase (similar to but not identical with cytochrome oxidase, cf. Borei, 1945) is the same in unfertilized and fertilized eggs, in the former, the enzyme is not saturated with substrate, owing to a block of some kind in the carrier chain. From the experiments with sodium azide and *p*-phenylenediamine, Stannard (1939) also assumed that the cytochrome oxidase of the frog muscle functions in the active but not in the resting state.

The experiments reported in this paper were therefore designed to investigate the possibility that the cytochrome-cytochrome oxidase system is involved in the respiratory Dilution Effect in sea-urchin spermatozoa.

MATERIALS AND METHODS

Spermatozoa of the sea-urchin, *Hemicentrotus pulcherrimus*, were used throughout. The semen was centrifuged for 5 min. at 3,000 r.p.m. The packed sperm was then diluted with filtered sea water. The manometric technique was similar to that described in the previous paper (Mohri, 1956), using vessels of about 20 ml. capacity and at 20° C.

Dpphd was usually employed as substrate, but *p*-phenylenediamine (pphd), hydroquinone, ascorbic acid and cysteine were also examined. All reagents were made up immediately before use in filtered sea water. The pH was adjusted to 8·2. Since no cytochrome *c* was added, the reaction depended on the intracellular cytochromes only.

RESULTS

Effect of dpphd on dense and dilute sperm suspensions

In contradistinction to the situation with other substrates, the catalytic oxidation of dpphd through cytochrome oxidase does not require addition of cytochrome *c* (Borei & Renvall, 1949). In the present experiments, dpphd was introduced in the main chamber, and sea water was added up to 2·5 ml. Then 0·5 ml. of very dense sperm suspension was pipetted into the side arm and poured into the main chamber after 10 min. temperature equilibration. The converse procedure (0·5 ml. of dpphd in the side arm, tipped after equilibration) was also tried, but little difference was observed between them. Experiments with other reagents were run mostly with the former technique, unless otherwise mentioned.

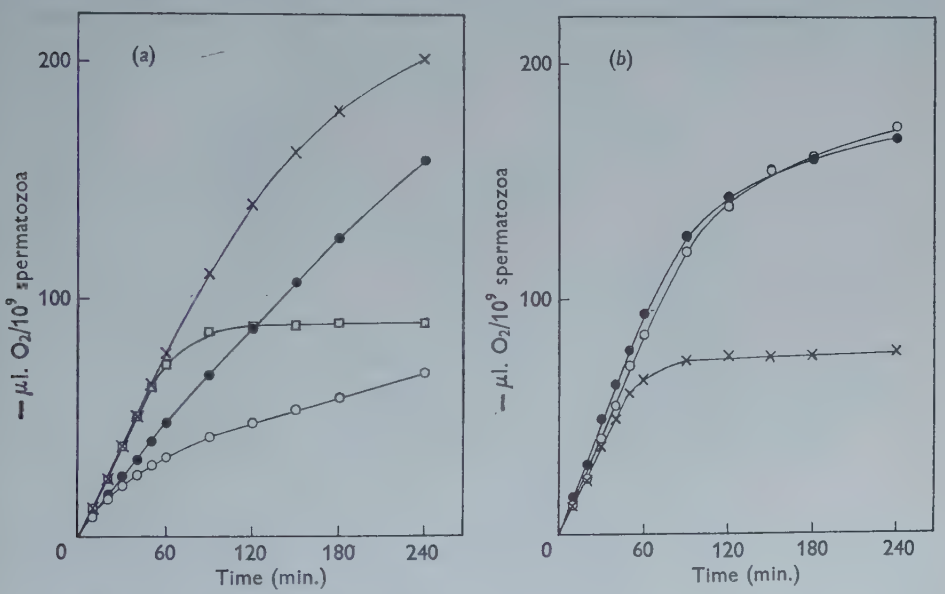

Fig. 1. Effect of dimethyl-*p*-phenylenediamine (dpphd) on O_2 uptake of sea-urchin spermatozoa, *Hemicentrotus pulcherrimus*. Dilution (*a*) 1/20; (*b*) 1/200. Final concentrations of dpphd: ○, 0; ●, 10^{-5} M; ×, 10^{-4} M; □, 10^{-3} M.

The effect of dpphd on the oxygen uptake of dense suspensions (1/20 or $2{\cdot}5 \times 10^9$ spermatozoa per ml.) is shown in Fig. 1*a*. A marked increase in oxygen uptake after the addition of dpphd is readily recognizable over the range of 10^{-5} to 10^{-3} M. Since the autoxidation of dpphd in sea water is negligible, the observed increase in oxygen uptake is not due to a catalytic action by copper ions in sea water. The respiration with dpphd proceeds at a constant rate during the first hour. The maximum rate is reached in the range from $3{\cdot}2 \times 10^{-5}$ to 10^{-3} M, though the latter concentration causes early death of the spermatozoa, which is reflected in a reduction in O_2 uptake after the first hour. The maximum rate is higher than the initial

rate of the control. With other respiratory stimulants such as 2,4-dinitrophenol (Mohri, 1956) or copper and zinc ions (Table 2; cf. Rothschild & Tuft, 1950), the effect does not become apparent until after about 1 hr.

The results obtained in dilute suspension (1/200 or $2{\cdot}5 \times 10^8$ spermatozoa per ml.) are illustrated in Fig. 1*b*. The addition of dpphd at concentrations of 10^{-5} and 10^{-4} M, which stimulate oxygen uptake of dense suspensions, has practically no effect on dilute suspensions, although for the same concentration of dpphd the spermatozoa die earlier in the dilute suspension. Furthermore, from a comparison of the curves in Fig. 1 *a* and *b*, it can be noted that the oxidation rate with sufficient amount of dpphd in the dense suspension is almost comparable to that in the dilute one. These facts suggest that in dense suspension cytochrome oxidase functions only partially, owing probably to lack of saturation with substrate. Dpphd short-circuits this obstacle and brings the enzyme almost to a maximum activity, while in dilute suspension this situation is already attained leaving no room for dpphd to exert its effect.

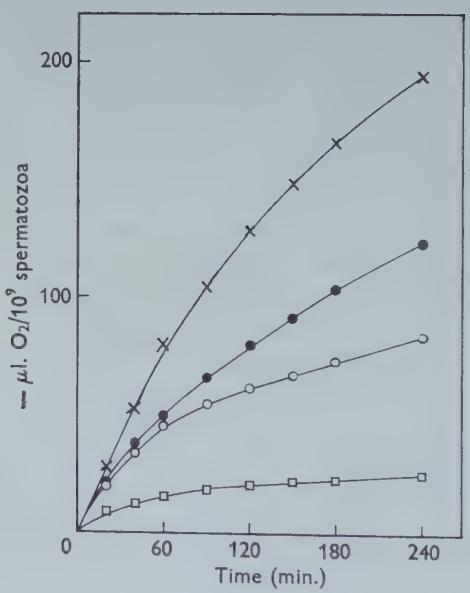

Fig. 2. Effect of ascorbic acid on O_2 uptake of sea-urchin spermatozoa, *H. pulcherrimus*. Dilution 1/20. Final concentrations of ascorbic acid: ○, o; ●, 10^{-4} M; ×, 10^{-3} M; □, 10^{-2} M.

Effect of other substrates. The effects of other substances which can be oxidized through the cytochrome-cytochrome oxidase system were examined in dense suspension (1/20). Autoxidation of these substances (except hydroquinone) was negligible in sea water in the range of concentration used. Among them, pphd has a very similar stimulating effect to that of dpphd on the respiration of the sperm suspension (see also Fig. 4). The magnitude of oxygen uptake at the maximum stimulation is also the same as that found with dpphd, indicating a 100% mobilization of the enzyme. As shown in Fig. 2, a similar result is obtained with ascorbic

acid at 10^{-3} M. At 10^{-2} M, however, ascorbic acid suppresses O_2 uptake from the beginning. In the case of hydroquinone, on the other hand, an extraordinary autoxidation occurs in sea water. According to Runnström (1932) and Örström (1932), if hydroquinone is added to sea water in which sea-urchin eggs are suspended, the hydroquinone acts as a true hydrogen carrier and no autoxidation occurs. Hydroquinone causes no increase in oxygen uptake of sea-urchin spermatozoa, but inhibits respiration at 10^{-3} and 10^{-2} M, unlike the previously mentioned substrates. The life-span of the spermatozoa is shortened by this substance. Zittle & Zitin (1942) also failed to detect cytochrome oxidase activity of homogenized bull spermatozoa with hydroquinone as a substrate. A marked spermicidal power of quinones was reported by Baker (1932).

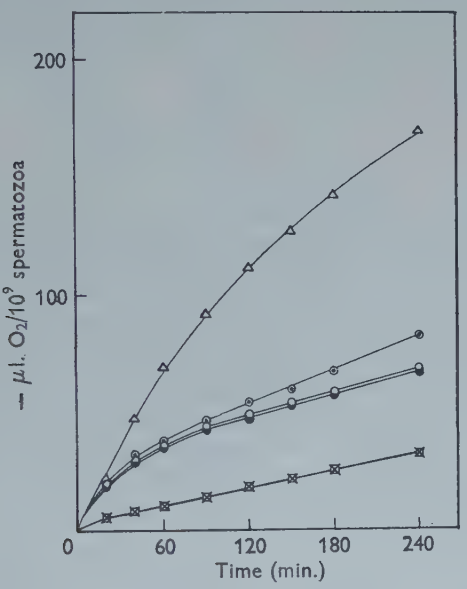

Fig. 3. Effect of cysteine on O_2 uptake of sea-urchin spermatozoa, *H. pulcherrimus*. Dilution 1/20. Final concentrations of cysteine: ○, ○; ●, 10^{-5} M; ×, 10^{-4} M; □, 10^{-3} M; ⊙, 10^{-2} M; △, $3{\cdot}2 \times 10^{-2}$ M.

As illustrated in Fig. 3, the results obtained with cysteine are not simple. When 10^{-5} M cysteine is used there is no change in oxygen uptake of the sperm suspension. From 10^{-4} to 10^{-3} M the initial oxidation rate is reduced to about the same level as that of the steady low respiration of the control after the initial burst. Above 10^{-2} M an increase in oxygen uptake can be observed. The stimulating action of cysteine and glutathione, both at 10^{-2} M, on oyster sperm respiration was reported by Humphrey (1950). Tyler (1953), on the other hand, found that amino acids including cysteine produce a prolongation of the life-span of sea-urchin spermatozoa. He suggested that this effect is due to the ability of amino acids to bind heavy metals, especially copper and zinc, present in the dilution medium. In the presence of amino acids or of other chelating agents, the initial rate of oxygen consumption is distinctly lower than that of the controls (Tyler & Rothschild, 1951; Tyler, 1953;

Rothschild & Tyler, 1954; see also Table 2). The increase in life-span of spermatozoa in the presence of cysteine is also confirmed in the present material (Table 1). This effect is obtained both at $3{\cdot}2 \times 10^{-2}$ M, which stimulates oxygen uptake, and at $3{\cdot}2 \times 10^{-4}$ M, which reduces the initial rate, although the latter concentration is more favourable than the former in keeping the spermatozoa alive.

Table 1. *Effect of cysteine on fertilizing capacity of sea-urchin spermatozoa,* Hemicentrotus pulcherrimus

(Fertilizing capacity was tested after 4 hr. incubation at 20° C. of $2{\cdot}5 \times 10^8$ sperm per ml. suspension. To 5 ml. of variously diluted suspensions approximately 500 unfertilized eggs were added. Numbers represent percentage of fertilized eggs.)

No. of sperm/ml.	Concentration of cysteine in sea water			
	0	$3{\cdot}2 \times 10^{-2}$ M	$3{\cdot}2 \times 10^{-4}$ M	$3{\cdot}2 \times 10^{-6}$ M
$9{\cdot}8 \times 10^6$	10	100	100	10
$2{\cdot}0 \times 10^6$	1	98	100	2
$3{\cdot}9 \times 10^5$	0	73	93	0
$7{\cdot}8 \times 10^4$	0	33	54	0

Effect of azide on oxidation of dpphd and of pphd

In the previous paper (Mohri, 1956) the respiration of sea-urchin spermatozoa was reported to be separable into two fractions, azide-sensitive and azide-insensitive, and that dilution initiates the former fraction. Since azide is one of the most powerful inhibitors of cytochrome oxidase, and furthermore since cytochrome oxidase activity is higher in dilute than in dense suspensions, it is quite justifiable to suppose that the azide-sensitive respiration is effected through cytochrome oxidase. To confirm this possibility the influence of azide on the enhanced respiration with substrates was examined. In this experiment, 0·5 ml. of a mixture of azide and dpphd or pphd was put in the side arm and was tipped into the main chamber after equilibration. As shown in Fig. 4, azide at 10^{-2} M is sufficient to eliminate completely the extra oxygen uptake (the effect of dilution as well as that of dpphd or pphd), leaving only respiration which coincides in magnitude with the azide-inhibited control respiration.

The increased oxygen uptake with ascorbic acid can also be inhibited by azide, but not quite to the level of the azide-stable residue of the control. The oxidation by cysteine, on the other hand, is inhibited to a lesser extent, suggesting that the increase in oxygen uptake found at high concentrations of cysteine does not go through the channel of cytochrome oxidase.

Effect of $ZnCl_2$ *and of metal-chelating agents on oxidation of dpphd*

The addition of copper or zinc to dense sperm suspensions can enhance oxygen uptake, and this is counteracted by the addition of metal-chelating agents such as ethylenediaminetetra-acetate (versene) and diethyldithiocarbamate (dedtc) (Rothschild & Tuft, 1950; Rothschild & Tyler, 1954). Since dilution brings about an increase in cytochrome oxidase activity, there is a possibility that the heavy

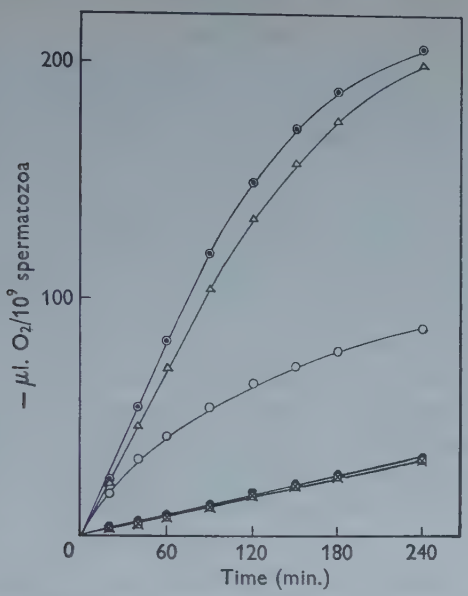

Fig. 4. Effect of sodium azide (10^{-2} M) on oxidation of dpphd (10^{-4} M) and pphd (10^{-4} M) in sperm suspension of sea-urchin, *H. pulcherrimus*. Dilution 1/20. ○, none; ⊙, dpphd; △, pphd; ●, azide; ×, dpphd+azide; □, pphd+azide.

Table 2. *Effect of $ZnCl_2$ and of chelating agents on respiration of the sea-urchin spermatozoa*, Hemicentrotus pulcherrimus, with and without dpphd

(Dilution was 1/20. The figures relate to the first hour at 20° C.)

Concn. of dpphd	$-\mu l.\ O_2/10^9$ spermatozoa					
	Control	$ZnCl_2$ 10^{-6} M	Control	Versene 10^{-3} M	Glycine 5×10^{-2} M	Dedtc 10^{-4} M
0	44·9	45·2	51·6	10·1	9·1	10·5
10^{-5} M	56·4	53·9	—	—	—	—
10^{-4} M	81·8	81·6	73·6	19·9	52·0	14·3

metals act directly on the cytochrome-cytochrome oxidase system. By contrasting the effects of $ZnCl_2$ and chelating agents, versene, dedtc and glycine, on the oxidation with and without dpphd in dense suspension (1/20), the results turned out to be as described in Table 2; as already mentioned, $ZnCl_2$ at 10^{-6} M without dpphd, which would cause a marked increase in oxygen uptake if added after a decline, had no effect on the initial phase of respiration, while all chelating agents suppressed the initial rate of normal respiration to a low level. These facts fit in with the above supposition. However, the situation becomes more complicated after further experimentation. Addition of excess zinc ions to dpphd 10^{-5} M, under conditions of submaximal oxidation, fails to improve the rate. Furthermore, the chelating agents do not inhibit the extra oxygen uptake in the presence of dpphd. This might mean that the point where the heavy metals are concerned is not the terminal oxidase system, but may be some other link in the oxidation chain.

DISCUSSION

The results reported in this paper seem to support the view that the cytochrome-cytochrome oxidase system is involved in the respiratory Dilution Effect in sea-urchin spermatozoa. Rothschild (1948) also observed that the inhibition of sea-urchin sperm respiration by carbon monoxide is greater in dilute than in dense suspensions. Cytochrome oxidase, however, is located at the terminal point in the oxidation chain and the activation of the enzyme will, on this view, be an ultimate result. This may suggest the existence of some other mechanism responsible for saturating the terminal oxidase with substrate. In a sense, the latter can be considered as the real cause of the Dilution Effect.

As described above, copper and zinc have a stimulating effect on the respiration of sea-urchin spermatozoa (Rothschild & Tuft, 1950; Mohri, 1956). Since copper and zinc are normal constituents of sea water and the increase in oxygen uptake on dilution is reported to depend on the amount of these metals present in sea water (Rothschild & Tuft, 1950), it is quite likely that these metals play a primary role in the mechanism of the Dilution Effect. The possibility that they directly affect cytochrome oxidase was examined, but without success. Barron, Nelson & Ardao (1948) reported that very low concentrations of sulphydryl-binding substances such as monoiodoacetate, *p*-chloromercuribenzoate and $CdCl_2$ accelerate the respiration of sea-urchin spermatozoa, whereas higher concentrations cause an inhibition. This is interpreted as showing that the addition of such SH-blocking reagents in small concentrations will cause the removal of the soluble SH groups regulating cellular respiration, resulting in an increase in oxygen uptake; with higher concentrations, however, SH groups in the protein moiety of enzymes will be destroyed, which results in inhibition of respiration. Copper and zinc will also serve as SH-binding reagents, and in fact the respiration of sea-urchin spermatozoa can be accelerated at 10^{-6} M or 10^{-5} M of both metals, though it is inhibited by 10^{-4} M and stronger solutions. The following scheme, then, might be applicable to the mechanism of the Dilution Effect in sea-urchin spermatozoa. When shed and diluted with sea water, the spermatozoa come in contact with copper and zinc ions in sea water. These metals combine with the soluble SH groups which regulate the oxidation rate of cytochrome *c*, eliciting the full activation of cytochrome oxidase. This scheme, however, is only tentative and further study is needed concerning the soluble SH groups.

SUMMARY

1. The activity of cytochrome oxidase in sea-urchin spermatozoa (*Hemicentrotus pulcherrimus*) was studied in relation to the Dilution Effect, using dimethyl-*p*-phenylene-diamine (dpphd), *p*-phenylenediamine (pphd), hydroquinone, ascorbic acid and cysteine as substrates.

2. All substrates except hydroquinone cause a marked rise in oxygen uptake in dense sperm suspensions (1/20). It is shown that the maximum rate obtained with a sufficient amount of dpphd in dense suspensions is almost comparable with the normal respiratory rate in dilute suspensions (1/200). The oxygen uptake of dilute suspensions is not affected by the addition of dpphd.

3. The increase in oxygen uptake caused by dpphd and pphd is completely eliminated by sodium azide (10^{-2} M).

4. From the results of 2 and 3, it can be inferred that cytochrome oxidase exhibits its maximum activity only in dilute suspension, probably as a result of substrate saturation.

5. Heavy metals, copper and zinc, do not seem to affect the terminal oxidase system directly, but some other part of the oxidation chain, such as the soluble SH groups.

6. The effect of cysteine is rather complicated. In the range from 10^{-4} to 10^{-3} M, cysteine causes a reduction in the initial respiratory rate as already observed with other amino-acids, but above this range, it exerts a marked stimulatory effect on oxygen uptake.

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ACTIVATION OF EGGS BY HYPOTHERMIA IN RATS AND HAMSTERS

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(With Plates 4 and 5)

INTRODUCTION

Previous work on the activation of mammalian eggs by cold has involved treatment of the eggs *in vitro*, followed by replacement in the Fallopian tube (Chang, 1954), or chilling of the eggs *in vivo*, by the application of cold water or ice to the tube (Pincus & Shapiro, 1940; Thibault, 1949; Austin & Braden, 1954*a*, *b*). In the rat, only the latter method has been employed. As a result of the cold-shock, rat eggs regularly extruded the second polar body and occasionally showed reformation of a nucleus and early cleavage. Since a variety of anaesthetics also induced activation of eggs in rats, it was suggested that the effect was due, in part at least, to the development of a tissue anoxia (Austin & Braden, 1954*a*).

No observations appear to have been reported on the artificial activation of the hamster egg, though some instances of spontaneous activation have been seen (Austin, 1956).

The development of methods for inducing profound hypothermia in rats and hamsters provides an opportunity for investigating the activating influence of cold without interference from anaesthesia or surgical operation. The observations described in the present communication show that eggs may be activated at temperatures well within the range that can be tolerated by the animal body as a whole.

METHODS

Rats were selected for experiment when they provided fully cornified vaginal smears on examination between 9.0 and 10.0 a.m., and hamsters when they gave smears of nucleated epithelial cells at this time. Thus, in both species, freshly ovulated eggs were present in the Fallopian tubes when hypothermia was induced. Some rats and hamsters were kept under controlled illumination, so that ovulation occurred chiefly between 12.0 noon and 4.0 p.m. (Austin & Braden, 1954*c*; Austin, 1956)—these animals were used in experiments involving chilling before ovulation. For the other animals it was assumed from the results of earlier work (Graves, 1945; Ward, 1946; Runner, 1947; Everett, 1948; Pederson, 1951; Austin & Braden, 1954*c*) that ovulation took place chiefly between 12.0 midnight and 4.0 a.m.

Hypothermia was induced by the methods described by Andjus & Lovelock

(1955) and Andjus & Smith (1955) for the rat, and by Smith, Lovelock & Parkes (1954) and Smith & Lovelock (1955) for the hamster. By these means body temperatures down to $-6°$ C. in hamsters and 0° C. in rats can be obtained with subsequent recovery. For body temperatures above 0° C. the chilling procedure was curtailed and the temperature maintained at the desired level for such a period that the duration of the experiment was similar to that involved by the unabridged method. In this way all animals experienced subnormal temperatures for approximately the same time, although the depth of hypothermia differed.

The effect of hypoxia alone was investigated by keeping the animal in a closed jar of about 800 ml. capacity, at room temperature, until respiration almost ceased (about 15–20 min.). On removal from the jar, the animals, particularly the hamsters, were found to recover rapidly and without assistance.

Eggs were obtained by dissecting the Fallopian tubes under normal saline solution and examined with a phase-contrast microscope. For study of chromosomes and spindle morphology the eggs were fixed, while still compressed under a cover-slip, with a 5% solution of acetic acid in absolute alcohol and stained with an aqueous solution of toluidine blue. Estimates of nuclear and nucleolar volumes were made on living eggs by the means previously described (Austin, 1952).

OBSERVATIONS

(1) *Rats*

Five rats, that had been kept under controlled illumination, were chilled to body temperatures between 0 and 1° C. about 0–4 hr. before the time of ovulation (Table 1). When killed the following day these rats yielded 45 eggs, of which only 2 (4%) showed evidence of activation: a polar body, presumably the second, for the first rarely persists (Austin & Braden, 1954*a*), had been extruded but the chromosomes had become scattered.

Table 1. *The incidence of activation in rat eggs as a result of induced hypoxia or hypothermia*

Treatment	No. of rats	Total eggs	Activated eggs	
			No.	%
Hypoxia	8	109	39	36
Hypothermia, 17–31° C.	8	101	25	25
Hypothermia, 0–1° C. (after ovulation)	6	75	75	100
Hypothermia, 0–1° C. (before ovulation)	5	45	2	4

All the remaining rats were kept under normal lighting conditions, and the experimental treatments were applied about 8–12 hr. after ovulation.

Twelve rats were subjected to a body temperature of between 0·0 and 1·0° C., and six of them were killed about 3 hr. later. These provided 75 eggs, in all of

which the second polar body had been extruded: all the eggs were therefore classed as activated (Table 1). The other six rats were killed the following day; 65 eggs were recovered, none of which exhibited well-formed nuclei. Eight eggs had undergone fragmentation, and 2 of these superficially resembled fertilized 2-cell eggs, but they possessed only scattered subnuclei in the cytoplasm in place of normal nuclei.

In eight rats body temperatures between 17 and 31° C. were induced; of 101 eggs recovered later 25% were found to be activated. Finally, eight rats were subjected to hypoxia alone, the body temperature remaining within 3° C. of the normal. These animals yielded 109 eggs of which 36% were activated.

(2) *Hamsters*

(*a*) *Spontaneous activation*

Six untreated hamsters, killed 8–12 hr. after ovulation, provided 60 eggs in which the second maturation division was in metaphase and only one polar body was visible. In none of these eggs, therefore, was there any evidence of activation (Table 2).

Table 2. *Hamster eggs showing spontaneous activation when recovered at different times after ovulation*

Time after ovulation (hr.)	No. of hamsters	Total eggs	'Zonas'	Activated eggs				
				In meiosis	With nuclei	With cleavage spindle	2-cell eggs	% activated
8–12	6	60	0	0	0	0	0	0
13–17	6	58	1	5	5	0	0	17
18–22	5	39	1	2	28	0	0	77
24–28	5	46	3	0	17	18	0	76
30–34	4	31	1	0	12	13*	0	81(?)
36–40	4	37	4	0	6	?	1	?
58–62	4	23	4	0	6†	0	0	?

* Most eggs showing break-up of spindle and chromosome scatter.
† All nuclei abnormal in appearance.

From six hamsters killed 13–17 hr. after ovulation, 58 eggs were recovered, of which 10 (17%) showed evidence of activation; 5 eggs exhibited phases of the second meiotic division and 5 had two polar bodies and nuclei similar to those shown in Pl. 4, figs. 1–3.

A much higher incidence of activation, namely 77%, was found among the eggs recovered 18–22 hr. after ovulation. Most of the eggs had a nucleus and two polar bodies, but there were 2 eggs that still displayed the second meiotic division. The nuclei were usually well formed and resembled those shown in Pl. 4, figs. 3 and 4, but in some eggs they did not look quite normal in that the nuclear boundary was indistinct and the nucleoli were gathered at the centre of the nucleus.

A similar incidence (76%) of activated eggs was noted at 24–28 hr. after ovulation; of these eggs about half had formed what appeared to be the metaphase of a first

cleavage spindle, the remainder still having nuclei. No anaphase or telophase stages were seen. While a number of the spindles were well formed, like that shown in Pl. 5, fig. 6, most were poorly developed, and in some eggs the chromosomes were already somewhat scattered (Pl. 5, fig. 10). In some of the unactivated eggs also, the maturation spindles were beginning to break up (Pl. 5, fig. 9). Disorganization of spindles was more common in the eggs recovered at the next period of observation, 30–34 hr. after ovulation, and in many instances the chromosomes were so widely scattered that it was uncertain whether they were derived from maturation or cleavage spindles. Consequently, the figure given for the incidence of activation in this group, 81 %, may be too high.

In the next two groups of hamsters, killed 36–40 and 58–62 hr. after ovulation, chromosome scatter was much more common and in none of the eggs could a cleavage spindle be positively identified. Many of the eggs, indeed, apparently contained no chromatic material at all. Nuclei, when present, were abnormal, grossly so in the eggs seen at 58–62 hr. Amongst all this evidence of abnormality there was one 2-cell egg (Pl. 5, fig. 8) which closely resembled a fertilized egg in its general form and in the appearance of its nuclei.

Among the 68 spontaneously activated eggs containing nuclei, and excluding those recovered at 58–62 hr. with grossly abnormal nuclei (Table 2), there were 15 binucleate eggs (Pl. 4, fig. 5), the rest being mononucleate. It was noteworthy that the binucleate eggs with the best-formed nuclei were those that had a single polar body—often the two nuclei were very similar to male and female pronuclei. In binucleate eggs with two polar bodies the nuclei were small and ill-formed.

Many of the eggs recovered from untreated hamsters, including the activated eggs, were characterized by the presence of one or more cytoplasmic vacuoles, more especially at the later periods. Vacuoles are seldom seen in fertilized eggs. In none of the eggs from untreated hamsters was there evidence of fragmentation, such as commonly occurs in unfertilized rat eggs (Austin, 1949).

(*b*) *Induced activation*

Six hamsters were chilled before ovulation; they had been maintained under controlled illumination, and the chilling procedure was begun at 9.30–10.0 a.m. on the day of ovulation. Minimum temperatures were reached about noon to 12.30 p.m. and rewarming was virtually complete by 1.0–1.30 p.m. Minimum temperatures varied between 0 and 10° C. (two hamsters at 0·5° C., two at 5° C., and two at 10° C.). The animals were killed at 5.0 p.m. (1–5 hr. after ovulation); they yielded a total of 46 eggs, all of which had a single polar body and the second maturation spindle in metaphase (Table 3). None of the eggs, therefore, had been activated by the chilling.

Hamsters with freshly ovulated eggs in the Fallopian tube were chilled to body temperatures of 0–1° C., 3–5° C., 8–16° C. or 20–25° C. The chilling procedure began about 10.0 a.m. and rewarming was in progress by 1.0 p.m. The animals were killed at 5.0 p.m. (13–17 hr. after ovulation). A high incidence of activation was found after chilling to 0–1° C. (80 % activation), to 3–5° C. (81 % activation)

and to 8–16° C. (86% activation) (Table 3). Body temperatures of 20–25° C., however, gave rise to only 34% activation. Hypoxia at room temperature, which was associated with a negligible fall in body temperature (to 36–38° C.), did not lead to activation in any of the eggs.

Table 3. *Eggs recovered at different times from hamsters subjected to hypothermia or hypoxia*

Time of killing after ovulation (hr.)	Time of treatment before (−) or after (+) ovulation (hr.)	Body temperature (° C.)	Number of hamsters	Total eggs	'Zonas'	Activated eggs				
						In meiosis	With nuclei	With cleavage spindle	2-cell eggs	% activated
1–5	−0–4	0–10	6	46	0	0	0	0	0	0
13–17	+6–10	0–1	6	60	0	3	45	0	0	80
13–17	+6–10	3–5	6	58	1	4	43	0	0	81
13–17	+6–10	8–16	6	51	2	8	36	0	0	86
13–17	+6–10	20–25	4	38	0	0	13	0	0	34
13–17	+6–10	36–38 (hypoxia)	5	42	0	0	0	0	0	0
30–40	+6–10	0–16	6	73	9	1	2	55	2	82
54–62	+6–10	5–16	6	43*	12	0	0	28†	0	?

* Includes 3 eggs that were fragmented.
† Eggs showed all degrees of spindle break-up and chromosome-scatter.

The activated eggs seen at 13–17 hr. after ovulation included a few showing stages of the second maturation division, but the majority had nuclei and two polar bodies. All stages of nuclear development were seen (Pl. 4, figs. 1–4), and at each the induced nucleus closely resembled, both in size and form, a normal female pronucleus. Dimensions of six of the larger nuclei were measured: the mean nuclear volume was 2790 μ^3 (range 2261 to 3222 μ^3), total nucleolar volume 327 μ^3 (309–440 μ^3) and number of nucleoli 7·5 per nucleus (2–12).

Six hamsters that had been chilled to body temperatures between 0 and 16° C. were killed the following day. Of the 73 eggs recovered, 82% were activated (Table 3), the majority displaying the metaphase of a first cleavage spindle, as in Pl. 5, fig. 6. Again, no anaphase or telophase stages were seen. The spindles were nearly all normal in appearance, although they presumably had a haploid chromosome complement, since two polar bodies had been extruded. In one egg (Pl. 5, fig. 7) the chromosomes were conveniently displayed, and twenty to twenty-two chromosomes could readily be counted; this is approximately the haploid number ($2n = 44$). Two eggs were nucleated and one still showed the second meiosis. There were also two 2-cell eggs, such as that in Pl. 5, fig. 8, which closely resembled fertilized 2-cell eggs. In addition to the activated eggs there were 4 eggs in which activation had not apparently occurred, and 9 eggs that consisted only of the zona pellucida containing a small amount of granular debris (Pl. 5, fig. 11); these are referred to in Table 3 as 'zonas'. The cytoplasm had evidently been lost through a break in the zona.

A further six hamsters, similarly treated, were killed 54–62 hr. after ovulation (Table 3), and these yielded 43 eggs, most of which showed varying degrees of degeneration; three had undergone fragmentation. There were 28 eggs that showed scattered chromosomes, which could have originated from a cleavage spindle, but the disarrangement was often so bad that they could equally well have come from the second maturation spindle. In 3 eggs no chromatic material at all could be discerned. There were also 12 eggs that were classed as 'zonas', since all or most of the cytoplasm had been lost.

Of the 94 nucleated eggs recovered from chilled hamsters, there were only 3 binucleate eggs, resembling the egg shown in Pl. 4, fig. 5, the rest being mononucleate. All the nucleated eggs seen at 13–17 hr. after ovulation had two polar bodies; no decision could be made on the two eggs recovered at 30–40 hr. owing to the presence of much granular debris in the perivitelline space.

DISCUSSION

The hamster egg evidently has a strong tendency towards spontaneous parthenogenesis. In the present study nearly 80% of eggs from untreated hamsters were observed to have undergone activation within 24 hr. of ovulation. Moreover, the progress of the meiosis, and generally also the development of the nuclei, took place in a manner closely similar to that of the corresponding stages in fertilization. The time relations, too, were much the same. Comparison of the present data with data published previously on fertilization in hamsters (Austin, 1956) shows that the times required for the egg to pass from the stage of spermatozoon penetration or of spontaneous activation to the formation of a cleavage spindle are both about 13–17 hr.

The occurrence of spontaneous early parthenogenesis appears to be unknown in other mammals, except as a rare event. Unfertilized rat eggs were found sometimes to undergo fragmentation in such a way as superficially to resemble cleaving fertilized eggs, but both 1-cell and divided eggs nearly always showed decided abnormalities of nuclear and cytoplasmic structure (Austin, 1949). In a later communication (Austin & Braden, 1954*d*) it was reported that among several thousand eggs from rats, mice and rabbits there were only two (rat) eggs that exhibited spontaneous nucleus formation. The striking feature of the hamster eggs is that so many of them displayed a normal appearance in nuclear and cytoplasmic components at all stages up to and sometimes including the cleavage spindle.

At 24–28 hr. after ovulation and later, eggs showing apparent parthenogenesis became less common in the untreated hamsters. Most of the cleavage spindles seem to break up instead of passing into the mitosis, and only one 2-cell egg was seen. The tendency towards parthenogenetic development, then, ceases at this stage, or else some item normally contributed by the spermatozoon is required for further progress.

Both in rats and hamsters the eggs were found to be highly resistant to artificial activation whilst still in the ovary. Evidently the second meiotic division is

precluded in some way from following immediately upon the first. On the other hand, a high incidence of activation in rat and hamster eggs was obtained by chilling the whole animal after ovulation, though there were several points of difference between the two species. In the rat a body temperature of 0–1° C. was just as effective as the application of ice to the Fallopian tube (Austin & Braden, 1954*a*, *b*), both methods giving 100% activation. But whereas in the earlier experience about 10% of eggs developed nuclei or, at later stages, showed apparently normal cleavage, in the present experiments no such examples of early parthenogenesis were seen. Perhaps this was because with hypothermia a reduced temperature is maintained for a much longer time than is involved when local chilling is effected by placing ice against the Fallopian tube.

In the hamster, only about 80% of eggs were found to be activated, but this occurred with a wide range of temperatures (0–16° C.). The incidence of activation referable to chilling is actually less than 80%, for at least 17% of eggs would have undergone spontaneous activation. The true figure would be between 60 and 70%. In view of the large proportion of eggs that ultimately become activated spontaneously the action of hypothermia may therefore be regarded simply as the hastening of a change that is inevitable. Nucleus and spindle formation were in general better with artificial activation, but the prospects of more extensive parthenogenetic development through this means do not appear to have been improved at all. Indeed, signs of degeneration—the frequency of fragmented eggs and 'zonas'—are more in evidence than with spontaneous activation.

Hypoxia alone produced activation in 36% of rat eggs, and this supports the contention that a relative tissue anoxia is probably the immediate effective agent in the activation by anaesthetics and to some degree in the activation by cold also. However, the fact that chilling to 0–1° C. had a greater influence than hypoxia alone means that the low temperature itself was responsible for much of the effect. The activation obtained (25%) with body temperatures between 17 and 31° C. is presumably referable entirely to the hypoxia induced in the early phases of the chilling procedure.

Hamster eggs were unaffected by hypoxia as brought about by restricted air supply. This may well be due to the development of a less intense tissue anoxia in these animals. Certainly the hamsters recovered much more rapidly than the rats.

In general the nuclei developed in artificially activated hamster eggs more often resembled pronuclei in appearance than did the nuclei in spontaneously activated eggs. The nuclear and nucleolar dimensions may be compared with those of pronuclei at full development, as previously determined (Austin, 1955). Thus, the mean nuclear volume of induced nuclei was about 2800 μ^3, while that of larger (probably male) pronuclei was about 3100 μ^3 and of smaller pronuclei about 2700 μ^3. On the other hand, the mean total nucleolar volumes were about 330 μ^3 for induced nuclei, and about 180 μ^3 and 150 μ^3 for larger and smaller pronuclei respectively. It has been proposed for rat and mouse eggs that the male and female pronuclei compete for limited amounts of formative material available in the egg cytoplasm, that the male pronucleus has the greater affinity for this material, and that there is

also an innate restriction on nuclear size (Austin & Braden, 1955). In the hamster egg the pronuclei evidently have much the same affinity for formative material and the innate restriction, which only becomes operative when a single nucleus develops in an egg, is such as to maintain nuclear size and form within the range exhibited by pronuclei. As in the eggs of rats and mice the restriction on total nucleolar volume is less than on nuclear volume. Indeed, it is possible that the innate restriction on total nucleolar volume is not effective in the hamster egg, since the volume attained by the nucleoli in induced nuclei approached the theoretical maximum, namely about the sum of the corresponding values of male and female pronuclei. These conclusions are supported by unpublished data obtained by the author on three hamster eggs that were undergoing apparent early gynogenesis. Each egg contained a single large nucleus and a spermatozoon, the head of which was still in an early phase of its metamorphosis. Mean nuclear volume was about 3050 μ^3 and total nucleolar volume about 340 μ^3. Here again, nuclear volume is about that of a single male or female pronucleus, while nucleolar volume is approximately equal to the sum of the nucleolar volumes of the two pronuclei. It seems likely that the gynogenetic eggs originated with the occurrence of spermatozoon penetration after spontaneous activation. Probably, then, by the time the spermatozoon head was ready for conversion into a male pronucleus the egg nucleus had completed most of its growth and in so doing had taken up the available formative material from the vitellus. This would apply particularly to the material required for the growth of nucleoli, and consequently the potential male pronucleus would be handicapped for nucleus formation and precluded from developing nucleoli. Consistently, in the gynogenetic eggs seen, the sperm head had been transformed into a small vesicular structure devoid of nucleoli.

Among the spontaneously activated hamster eggs with nuclei there were more with two nuclei than among the artificially activated eggs: 15 eggs out of 68 (22%) as compared with 3 eggs out of 94 (3%). This is probably because spontaneous activation occurred later than artificial activation, allowing the second maturation spindle time to move inwards away from the surface of the egg. If this happened, two nuclei would be formed after the completion of meiosis, as the spindle would be too far from the surface to permit extrusion of the second polar body. The formation of two female pronuclei in *Triturus* eggs after hot-shock treatment has been shown to occur by such a mechanism (Fankhauser & Godwin, 1948).

SUMMARY

Hamster eggs appear unique among known mammalian eggs in that nearly 80% were found to undergo spontaneous activation, extruding the second polar body, within 24 hr. of ovulation. Often the eggs showed spontaneous early parthenogenesis—the development of one or occasionally two nuclei and the formation of a haploid first cleavage spindle took place in a manner that closely resembled the corresponding stages in fertilization. Time relations, too, were much the same. Rarely, however, does the cleavage spindle pass into mitosis, but instead it breaks

up and the chromosomes become scattered. Only one normal-looking 2-cell egg was found.

Rat and hamster eggs were very resistant to activation by hypothermia while still in the ovary. After ovulation, however, 100% of rat eggs were activated by body temperatures between 0 and 1° C. and about 60–70% of hamster eggs by temperatures between 0 and 16° C. In view of the high incidence of spontaneous activation in hamster eggs, the effect of chilling in this animal can be regarded as merely that of hastening the change.

Hypoxia alone activated 36% of rat eggs. Relative tissue anoxia is therefore probably the immediate effective agent in activation by anaesthetics and is partly responsible for activation by low temperatures. Cold-shock itself, however, is evidently a more potent activator. Hamster eggs were not activated by hypoxia, probably because a less severe tissue anoxia was induced.

Rat eggs activated by hypothermia showed less tendency to early parthenogenesis than those activated by local chilling of the Fallopian tube.

Nucleus and spindle formation was rather better in artificially than in spontaneously activated hamster eggs, but the prospects of extensive parthenogenetic development did not seem in any way improved. Only two 2-cell eggs resembling fertilized eggs were seen.

Nuclei in activated hamster eggs achieved a volume similar to that of male or female pronuclei at full development. Total nucleolar volume was equal to the sum of the nucleolar volumes of male and female pronuclei.

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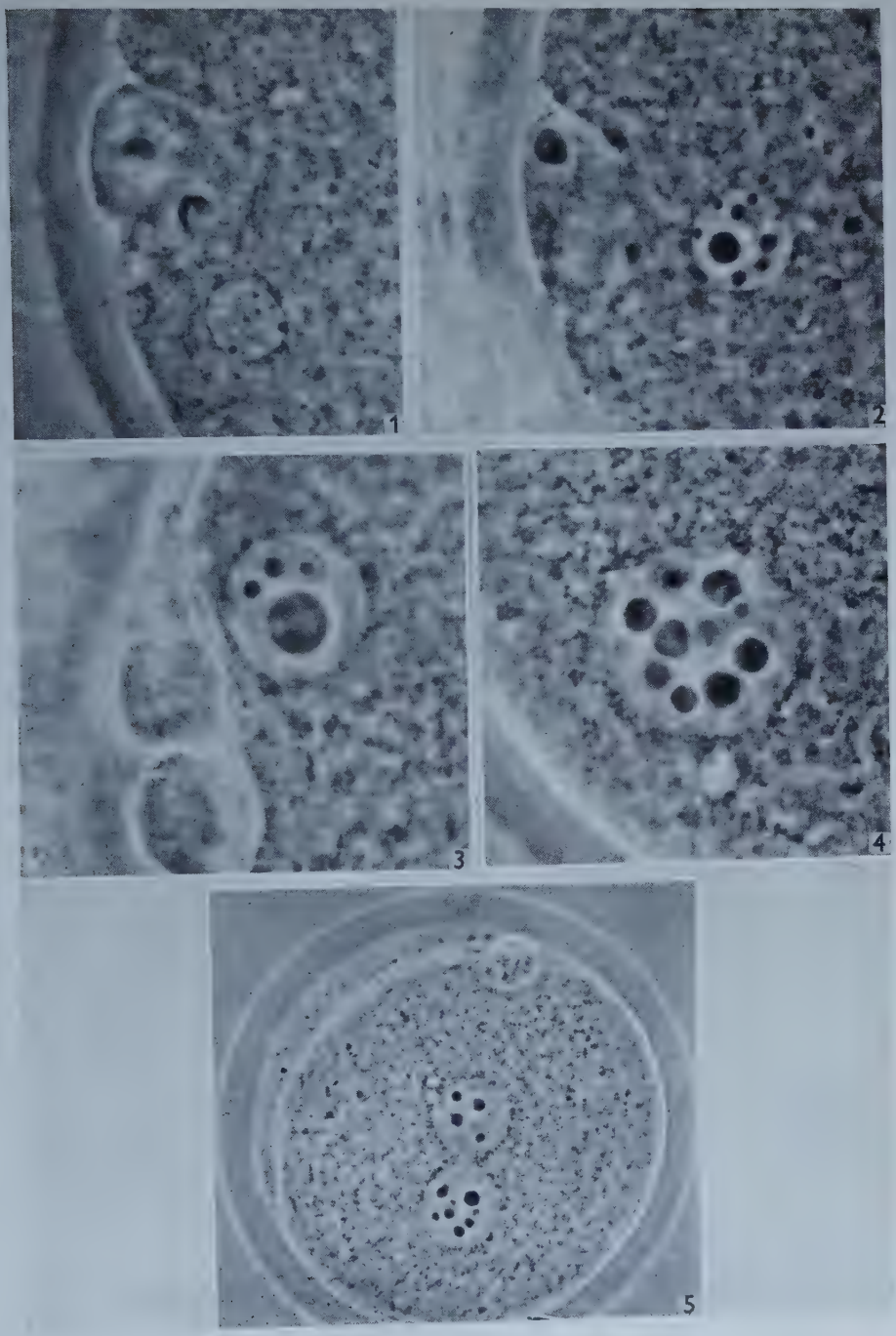

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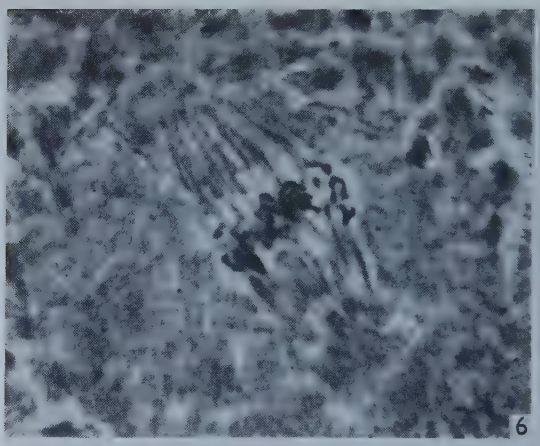

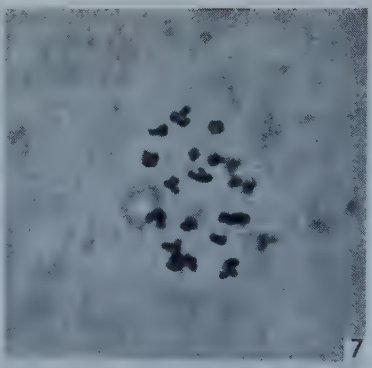

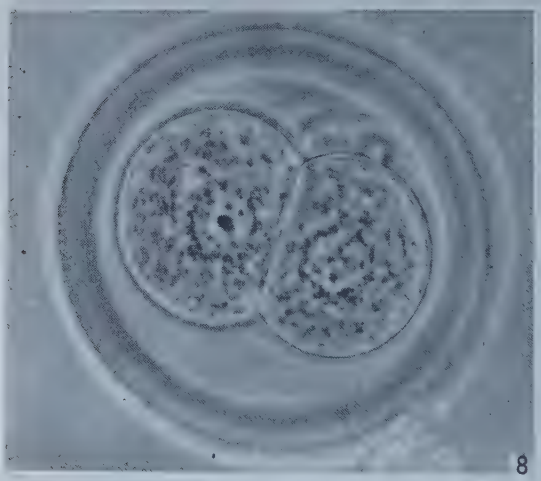

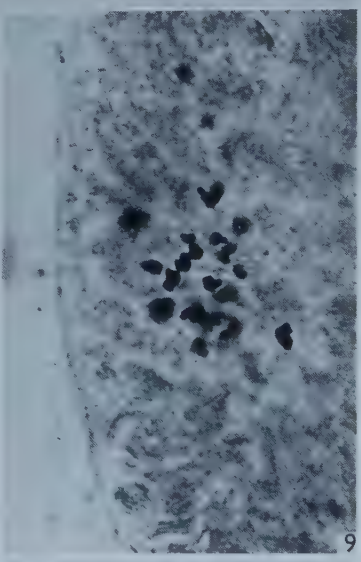

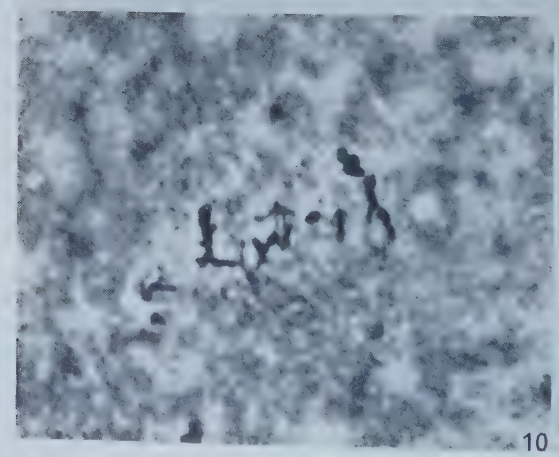

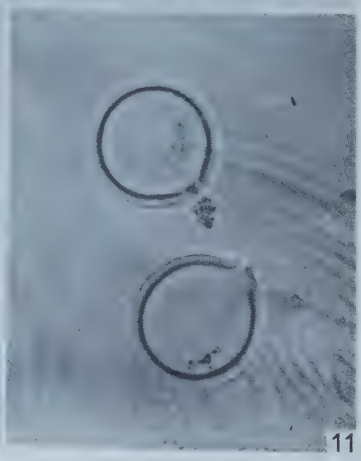

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EXPLANATION OF PLATES

The illustrations are of hamster eggs recovered from unmated animals. The photographs were all taken with a phase-contrast microscope, except for fig. 7. The eggs shown in figs. 6, 7, 9 and 10 were fixed and stained, whereas the others were photographed in the fresh state.

Plate 4

Figs. 1–4. Stages of the development of nuclei in eggs from hamsters that had been chilled. × 1000.
Fig. 5. A binucleate egg, with one polar body, from an untreated hamster. × 400.

Plate 5

Fig. 6. A normal-looking first cleavage spindle in an egg recovered from a chilled hamster killed 30–40 hr. after ovulation. × 1500.
Fig. 7. First cleavage metaphase chromosome group in an artificially activated egg showing twenty to twenty-two chromosomes ($2n=44$). × 1000.
Fig. 8. A 2-cell egg recovered 36–40 hr. after ovulation from an untreated hamster. In outward appearance and nuclear form it closely resembled a fertilized 2-cell egg. × 400.
Fig. 9. A scattered group of chromosomes from the break-up of a second maturation spindle. The egg came from an untreated hamster 24–28 hr. after ovulation. × 1000.
Fig. 10. The disintegrating cleavage spindle from an untreated hamster 30–34 hr. after ovulation. × 1500.
Fig. 11. Two eggs that consisted only of the zona pellucida with a small amount of granular debris inside. The rupture through which the greater part of the vitellus was lost is visible. These 'zonas' were recovered 54–62 hr. after ovulation from chilled hamsters. × 100.

EFFECTS OF HYPOTHERMIA AND HYPERTHERMIA ON FERTILIZATION IN RAT EGGS

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INTRODUCTION

The effects of heat and cold upon the course of fertilization in rats have been studied by immersing the Fallopian tube in water at 44·5–45·5° C., and by applying ice to the tube, both procedures being employed after ovulation (Austin & Braden, 1954*b*). The chief effects of the hot-shock treatment were a temporary inhibition of the second maturation division and a large increase in the incidence of polyspermy. Cold-shock, on the other hand, induced activation in all eggs, but this did not preclude their subsequent fertilization.

Since then a method has been published for lowering the body temperature of the rat to 0° C., with subsequent complete recovery (Andjus & Lovelock, 1955; Andjus & Smith, 1955). Moreover, it has been shown that a moderate hyperthermia was as effective as local hot-shock in increasing the incidence of polyspermy (Austin, 1955). Means are therefore available for investigating the influence of wide variations in temperature upon fertilization in the intact animal and without interference from anaesthesia. These methods have now been applied both before and after ovulation, and the results are described in this paper.

METHODS

Rats of the 'hooded' variety were used. They were kept in two rooms wherein the lighting was controlled. In one room natural illumination was supplemented during late autumn, winter, and early spring with artificial light, which was regulated by an automatic time switch so that the 'day' was never shorter than 15 hr. When in use the artificial light was switched on at 4.0 a.m. and off at 7.0 p.m. Rats kept in this room are referred to later as having been maintained under natural illumination. The other room was lit entirely by artificial light which was turned on by time switch at 4.0 p.m. and off at 7.0 a.m., throughout the year. Rats kept in this room are referred to as having been kept under controlled illumination. By these means it was possible to have available two groups of rats in which ovulation occurred approximately 12 hr. apart. The efficacy of this method of varying the time of ovulation has recently been demonstrated (Austin & Braden, 1954*a*). For the purpose of mating, male rats were put in with the females at the beginning of each week and removed each week-end.

The method used for inducing hypothermia was that set forth by Andjus & Lovelock (1955) and Andjus & Smith (1955); it is briefly as follows: the rat is placed in a jar of about $2\frac{1}{2}$ l. capacity designed to take a screw-on top with rubber seal. The top is screwed tight and the jar placed in a refrigeration chamber ($-2°$ C.). The combined effect of anoxia and cold produces a state of anaesthesia so that, at the end of about 2 hr., the rat is found to be lying motionless, except for regular breathing, and to have a colonic temperature of 16–20° C. The rat is removed from the jar and immersed in a dish containing ice and water. After about an hour in the ice bath the body temperature is generally about 0–1° C. Recovery is obtained by removing the rat from the bath and rewarming it, initially by radiant heat or diathermy, and later in a warm water-bath. The animals usually took $\frac{1}{2}$–$\frac{3}{4}$ hr. to return to body temperatures of 30° C. or more. In the experiments to be described different levels of hypothermia were induced, the desired body temperature being obtained by curtailing the chilling procedure at the appropriate point. After this the body temperature was kept within the specified range by leaving the rat, with a free supply of air, in one or other of three rooms ($-2°$, 4° and 15° C.) according to the need.

Hyperthermia was obtained by placing the rat in a box which had thermostatically controlled heating elements built into the floor. Adequate ventilation was provided by openings in the sides and top of the box. Observations were made on the air temperature in the box, and, in some experiments, on the relative humidity also. The rats were removed at intervals of $\frac{1}{2}$–1 hr. and their colonic temperature was taken. Box temperatures were controlled with reference to the rat's body temperature.

OBSERVATIONS

Time relations and incidence of ovulation and fertilization in untreated animals

(*a*) *Normal mating.* Female rats maintained under natural illumination were killed at 9.0 a.m. and 4.0 p.m. of the day on which a copulation plug was found. By 9.0 a.m., ovulation was evidently complete (10·9 eggs per rat), for no increase in the mean number of eggs occurred by 4.0 p.m. (Table 1). Spermatozoon penetration, however, was still in progress at 9.0 a.m., as only 61 % of eggs contained spermatozoa, compared with 94 % at 4.0 p.m.

The proportions of eggs containing two or more spermatozoa (16 and 20 %) did not differ significantly at the two times of slaughter. In most of these eggs the extra spermatozoa lay in the perivitelline space (supplementary spermatozoa); a few eggs, however, had two spermatozoa within the vitellus and were therefore polyspermic. No significant difference was seen in the proportions of polyspermic eggs recovered at 9.0 a.m. and 4.0 p.m. (1·3 and 2·0 %).

Rats kept under controlled illumination were killed at 2.0 p.m. and 4.0 p.m. of the day on which the copulation plug was found, and also between 11.0 a.m. and 4.0 p.m. on the following day (day 2). Ovulation had evidently not long been under way at 2.0 p.m. for there were only 2·3 eggs per rat killed at this time (Table 1). By 4.0 p.m. 6 of the 12 rats killed had eggs in the Fallopian tube and about half the

total number of eggs had been ovulated (5·7 eggs per rat). The figure for the total number of eggs to be ovulated was given by the rats killed on day 2 (10·5 eggs per rat) and this was essentially the same as the total number provided by the rats under normal illumination. Spermatozoon penetration was proceeding slowly during the afternoon of day 1, since only about 10% of eggs were found to be penetrated at both 2.0 and 4.0 p.m., and none of these eggs had extra spermatozoa. By day 2, however, spermatozoon penetration was virtually complete (97% of eggs penetrated). Nearly all these were 2-cell eggs, but some were in the later stages of pronuclear development. Some vestiges of the cumulus oophorus remained about the eggs of some rats, but mostly the eggs were denuded of surrounding cells. The incidence of eggs penetrated by two or more spermatozoa, and of polyspermic eggs, was much the same as in the rats kept under natural illumination. All the polyspermic eggs were dispermic.

Table 1. *Ovulation and spermatozoon penetration in untreated rats*

Illumination	Mating	Time of killing	Total rats	Rats with eggs	Total eggs	Mean eggs per rat killed	Penetrated eggs		Eggs penetrated by two or more spermatozoa		Polyspermic eggs	
							No.	%	No.	%	No.	%
Natural	Normal	9.0 a.m.	12	12	131	10·9	80	61	13	16	1	1·3
		4.0 p.m.	20	20	218	10·9	204	94	40	20	4	2·0
Controlled	Normal	2.0 p.m.	12	3	27	2·3	3	11	0	0	0	0
		4.0 p.m.	12	6	68	5·7	7	10	0	0	0	0
		Day 2	12	12	126	10·5	122	97	23	19	2	1·6
Natural	Delayed	5.0 p.m.	12	12	149	12·4	120	81	21	18	4	3·3
		Day 2	20	20	236	11·8	200	85	40	20	5	2·5

(*b*) *Delayed mating*. Female rats that had been kept under natural illumination were selected for fully cornified vaginal smears at 9.0 a.m. and placed with males until 10.0 a.m. Those that copulated were killed at 5.0 p.m. the same day or on the following day (day 2). Rats killed at 5.0 p.m. provided a mean of 12·4 eggs per rat, of which 81% had been penetrated by spermatozoa (Table 1). Essentially the same figures were recorded for the rats killed the following day. The proportions of eggs penetrated by two or more spermatozoa at both these times of slaughter were similar to those observed for the rats permitted normal mating. The figures for the proportion of polyspermic eggs, 3·3 and 2·5% respectively, were higher than those for normal mating but not significantly so. These eggs, too, were all dispermic.

Effect of hypothermia applied before and during the period of ovulation

In this experiment rats that had been maintained under controlled illumination were used and the effect of three levels of hypothermia was tested: 0–1°, 4–7°, and 16–24° C. In the first group the body temperature was kept within the range of 0–1° C. for about 15 min., but the entire procedure took about 4 hr. In the other

groups, also, the entire procedure lasted about 4 hr. but the body temperature stayed in the specified ranges for proportionately longer, namely between ¾ and 1 hr. at 4–7° C., and between 2 and 3 hr. at 16–24° C. All the tests were carried out between 11.0 a.m. and 5.0 p.m.

On the following day, the rats were killed between 11.0 a.m. and 6.0 p.m., and the eggs examined. Eggs from the rats that had been chilled to 16–24° C. or to 4–7° C. showed some effect from the treatment but this was not great. The number of eggs produced differed insignificantly from normal, and the incidence of spermatozoon penetration (71 and 80% respectively) was not much reduced (Table 2). In both groups the penetrated eggs were in the later stages of fertilization or had undergone the first cleavage—much as in the untreated rats. However, most of the cumulus oophorus remained about the eggs from one rat that had been chilled to 16–24° C. and from 4 rats chilled to 4–7° C. By contrast, of the eggs from rats chilled to 0–1° C., only 2% showed spermatozoon penetration, and all eggs had the greater part of the cumulus cell mass still about them.

Table 2. *Ovulation and spermatozoon penetration in normally mated rats chilled before and during the time of ovulation*

Temperature (° C.)	No. of rats	Total eggs	Mean eggs per rat killed	Penetrated eggs		Eggs penetrated by two or more spermatozoa	Polyspermic eggs
				No.	%		
0–1	11	113	10·3	2	2	0	0
4–7	6	61	10·2	49	80	4	0
16–24	6	56	9·3	40	71	8	1

Effect of hypothermia applied after ovulation

Female rats that had been kept under natural illumination were selected for fully cornified vaginal smears and placed with males at 9.0 a.m. for delayed mating. Those with copulation plugs at 10.0 a.m. were promptly subjected to a chilling procedure, which lasted 5–6 hr., and killed at 5.0 p.m. The body temperatures of the rats were reduced to some point between 16 and 33° C., which took 1–2 hr., and the attempt was then made to keep the animals' temperature constant for 4–5 hr. Unavoidably, however, the body temperature varied, sometimes by as much as ±5° C., from the original point selected. It soon appeared that the occurrence of spermatozoon penetration depended almost entirely upon the temperature reached at the top of the individuals' range of variation. Accordingly, the temperatures listed in Table 3 for rats killed at 5.0 p.m. represent the maximum temperatures achieved by the rats during the 4–5 hr. period. It is evident that, if the rat's body temperature did not exceed 30–32° C. during the experimental period, the eggs subsequently recovered at 5.0 p.m. showed no spermatozoon penetration. Rats in which the temperature exceeded 30–32° C. but not 33–34° C. yielded 16% of eggs penetrated, and those that passed above 34° C. gave 75% of eggs penetrated.

Estimates were made at 5.0 p.m. of the number of spermatozoa at the site of fertilization in eight rats in which the maximum body temperature lay in the range 19–33° C. and which did not provide any penetrated eggs. The mean number of spermatozoa counted was fifty-seven per Fallopian tube, range 17–287.

Table 3. *Ovulation and spermatozoon penetration in delay-mated rats chilled after the time of ovulation*

Time of killing	Temperature (° C)	No. of rats	Total eggs	Mean eggs per rat killed	Penetrated eggs		Eggs penetrated by two or more spermatozoa	Polyspermic eggs
					No.	%		
5.0 p.m.	19–29	6	80	13·3	0	0	0	0
	30–32	6	80	13·3	0	0	0	0
	33–34	5	61	12·2	10	16	1	0
	35–37·5	5	61	12·2	46	75	1	1
Day 2	25–29	6	68	11·3	49	72	13	2
	5–10	6	64	10·7	32	50	5	3

To determine whether the inhibition of spermatozoon entry was permanent, six rats, similarly selected and mated, were chilled to 5–10° C. The body temperature was kept in this range for at least an hour—the mortality was rather high when longer periods were tried. These animals were killed the following day and provided 64 eggs, of which 50% contained spermatozoa—5 eggs even had two or more spermatozoa. A further six rats were chilled so that their temperatures did not exceed 25–29° C. for 4–5 hr., and these, on slaughter the following day, gave 72% of penetrated eggs, including 13 eggs with two or more spermatozoa.

Effect of hyperthermia applied before and during the period of ovulation

Eleven female rats that had been kept with males under controlled illumination were selected on different occasions for the presence of a copulation plug at 9–10 a.m. and then placed in a heated box. The period of heating lasted 1–4 hr. according to the tolerance shown. An attempt was made to keep the body temperature constant at about 3° C. above normal, but inevitably the temperature varied. For most of the experimental period the body temperatures were above 40° C., but at some time all rats reached 41·5° C. or more. If the temperature rose to 43° C., which happened in two rats, the heating was stopped. Mean maximum temperature for the group was 42·2° C., range 41·5–43° C. All rats survived until the following day, when they were killed. They yielded a mean of 7·5 eggs per rat, of which 66% showed spermatozoon penetration (Table 4). Of the penetrated eggs 28% had two or more spermatozoa, including three polyspermic eggs. The polyspermic eggs were dispermic; all three came from the one animal, so that the figure of 5·6% is not a reliable estimate of the group. The penetrated eggs were undergoing apparently normal fertilization.

Table 4. *Effect of hyperthermia on ovulation and spermatozoon penetration*

Mating	Time of heating	No. of rats	Total eggs	Mean eggs per rat killed	Penetrated eggs		Eggs penetrated by two or more spermatozoa		Polyspermic eggs	
					No.	%	No.	%	No.	%
Normal	Before and during ovulation	11	82	7·5	54	66	15	28	3	5·6
Delayed	After ovulation	31	345	11·1	307	89	95	31	65	21·2

Effect of hyperthermia applied after ovulation

Female rats kept under natural illumination were delay-mated between 9.0 and 10.0 a.m. and then placed in a heated box. Altogether thirty-one rats were used, but not more than three animals were treated on any one day. During the heating periods for thirteen of these rats the relative humidity, as well as the air temperature of the box, were measured. The humidity varied between 30 and 68 %; usually it was high in this range in the early stages and fell during the course of the heating period. As with the previous group, body temperatures showed frequent variations in spite of attempts to keep them constant. Hyperthermia was induced for 1–4 hr. depending upon the tolerance shown by the animals. For most of the heating period the body temperatures were above 40° C. In two rats the temperature rose suddenly to 44° C., whereupon they were cooled under running cold water. Nine rats reached 43° C., and some of these were also cooled with water, as they showed signs of heat prostration. For the group as a whole the mean maximum temperature was 42·4° C., range 41·5–44° C.

All rats survived until the following day, when they were killed. A total of 345 eggs was obtained, of which 89 % had been penetrated by spermatozoa (Table 4). Of the penetrated eggs 31 % contained two or more spermatozoa, and this included 21·2 % of polyspermic eggs. Amongst the latter there were 9 trispermic, 7 tetraspermic and one pentaspermic eggs, the rest (48 eggs) being dispermic. Many of the eggs were abnormal, especially those that exhibited the higher orders of polyspermy. The commonest fault was nuclear fragmentation, though a number of eggs had also undergone cytoplasmic fragmentation. Trispermic eggs are rare: none was recovered from untreated rats in the present series, and only 3 were observed among 2987 penetrated eggs in a previous investigation (Austin & Braden, 1953). The higher orders of polyspermy do not seem to have been recorded before in mammalian eggs.

When the results were examined no distinct correlation could be found between the proportion of polyspermic eggs and the degree or duration of hyperthermia. It soon appeared, however, that the larger rats were the more likely to provide polyspermic eggs and accordingly, for the remainder of the experiment, the animals were weighed. The results were divided into two groups: those for 170–200 g. rats

and those for 100–130 g. rats. The proportions of penetrated eggs recovered from larger and smaller rats were about the same: 83 and 88% respectively (Table 5). However, the proportion of eggs containing two or more spermatozoa was much higher with larger rats, 41% as compared with 27%, and so was the proportion of polyspermic eggs, 34% as compared with 13%. A further calculation can be made, namely the number of eggs that became polyspermic, compared with the number of eggs that could have become polyspermic, because two or more spermatozoa had passed the zona pellucida. Here again the figure obtained for the larger rats, 84%, was much higher than that for the smaller rats, 50%. Moreover, of the 13 tri- and tetraspermic eggs recovered from the weighed rats, all but one, a trispermic egg, came from the larger rats.

Table 5. *Incidence of spermatozoon penetration and polyspermy in larger and smaller rats subjected to hyperthermia after ovulation*

Weight of rats (g.)	No. of rats	Total eggs	Mean eggs per rat killed	Penetrated eggs		Eggs penetrated by two or more spermatozoa		Polyspermic eggs	
				No.	%	No.	%	No.	%
170–200	9	108	12·0	90	83	37	41	31	34
100–130	12	120	10·0	105	88	28	27	14	13

DISCUSSION

Time relations of ovulation and spermatozoon penetration in untreated rats were a little different from those previously reported (Austin & Braden, 1954*a*). Compared with the earlier results, the present data show both ovulation and penetration, in the rats as a group, to be spread out over a longer period. Presumably this is because the work was done in another country—and consequently under different light, temperature and nutritional conditions—and with rats of a different strain. As already noted (Austin, 1955), the same reasons may explain the fact that the incidence of polyspermy after delayed mating of rats in London differed insignificantly from that following normal mating, whereas in Sydney this procedure resulted in an approximately sevenfold increase.

When normally mated rats were chilled before and during the expected time of ovulation there was no detectable effect upon the number of eggs ovulated and little reduction in the incidence of penetrated eggs, with body temperatures down to 4–7° C. When the body temperature was reduced to 0–1° C., however, there was almost complete absence of spermatozoon penetration. The rats were killed the day after the experimental treatment, well after the time of spermatozoon entry in untreated animals, so that penetration seemed to have been permanently inhibited. The explanation of this effect is not immediately obvious. The eggs when recovered were quite normal in appearance. A hypothermia of 0–1° C. will activate all eggs in rats if applied after ovulation, but not if applied before this event (Austin, 1956). In any case, activation by cold-shock need not preclude subsequent fertilization

(Austin & Braden, 1954*b*). It seems unlikely that rat spermatozoa within the female tract would not have been able to survive the very moderate chilling in view of the well-known tolerance shown by the spermatozoa of many other species of mammals to temperatures down to 0° C. *in vitro*. Furthermore, the 'capacitation' (Austin, 1951, 1952) of the spermatozoa would almost certainly have been completed before chilling began, since it probably takes only 2–3 hr. (Austin & Braden, 1954*a*) and mating in the rat occurs 6–12 hr. before ovulation (Boling, Blandau, Soderwall & Young, 1941). Consequently, it does not seem possible to ascribe the apparent inhibition of spermatozoon penetration to any recognizable change in the eggs or the spermatozoa. Now, the much better persistence of the cumulus oophorus about the eggs in rats chilled to 0–1° C. strongly suggests that ovulation had been delayed by the hypothermia. This could be the prime cause of failure of penetration if the length of delay exceeded the survival period of the spermatozoa. Perhaps a shorter delay would suffice when associated with a derangement of the tubal environment, such as may well be produced by temperatures near 0° C.

A more clear-cut but plainly temporary inhibition of spermatozoon entry was obtained by chilling rats after ovulation. Delayed mating occurred between 9.0 and 10.0 a.m., and it has been shown that, when coitus takes place after ovulation, spermatozoon penetration does not begin for at least 2 hr. (Austin & Braden, 1954*a*). In the present series, the chilling of the animals was started soon after 10.0 a.m. and, therefore, before spermatozoon penetration. The results show a distinct threshold at about 32–33° C., penetration occurring only if the body temperature exceeded this level. It is remarkable not only that the threshold is so distinct but that it is so little below normal body temperature. The transient nature of the inhibition is indicated by the comparatively high proportion of penetrated eggs (72 %) recovered from rats in which the body temperature did not exceed 29° C. during the chilling period and which were killed the following day. Even when rats were chilled to 5–10° C., 50 % of their eggs were found to have been penetrated on slaughter the next day. The inhibitory effect can hardly be ascribed to interference with the transport of spermatozoa to the site of fertilization, for the numbers of spermatozoa found here were quite comparable with the numbers previously observed in untreated animals at similar times after delayed mating (Braden & Austin, 1954). The most likely explanation seems to be that capacitation of spermatozoa could not take place, or was greatly slowed, at temperatures below 33° C.

The effect of hyperthermia on fertilization also differed according to its application before and during, or after, the time of ovulation. When hyperthermia was induced before and during the time of ovulation, there was evidence that it interfered with the process of ovulation—only 7·5 eggs per rat were recovered, as compared with 10·5 eggs in untreated animals. The proportion of penetrated eggs, too, was reduced from 97 to 66 %. This is possibly owing to disturbance of terminal maturation in the eggs, such maturation being apparently a necessary preliminary to spermatozoon penetration (Austin & Braden, 1954*a*). Consistently, there was little or no reduction in the rate of penetration when heating was applied several

hours after ovulation. On the other hand, with hyperthermia after ovulation the proportion of eggs penetrated by two or more spermatozoa and the proportion of polyspermic eggs were both increased, especially the latter. Indeed, the incidence of polyspermy observed in the larger rats (34%) is the highest yet reported for a group of rats.

Data on the proportion of eggs penetrated by two or more spermatozoa, and of polyspermic eggs, are of interest in that they provide an inverse measure of the activity of the 'zona reaction' (Braden, Austin & David, 1954) and block to polyspermy, respectively. There is good reason to believe that the 'zona reaction', like the block to polyspermy, is the product of a change initiated in the vitelline cortex by contact with a spermatozoon (Austin & Braden, 1956). Evidently, the processes involved in the 'zona reaction' are deleteriously affected by hyperthermia, whether induced before and during or after ovulation. On the other hand, the block to polyspermy involves processes that are much more sensitive to heat after ovulation. In fact the block to polyspermy seems to show more distinctly the effect of ageing. As already mentioned, under certain circumstances delayed mating alone will cause a large increase in the incidence of polyspermy. Hyperthermia apparently accelerates the change involved. Larger (older) rats heated after ovulation yielded a much higher proportion of polyspermic eggs (34%) than smaller rats (13%). This difference can hardly be due to the presence in the larger rats of more spermatozoa at the site of fertilization or of the existence of more favourable conditions for penetration, since the proportion of penetrated eggs differed insignificantly from that seen in the eggs from the smaller rats. It seems that older rats have inherently older eggs, or else that eggs, once ovulated, age more rapidly in older rats.

SUMMARY

The chilling of rats to body temperatures down to 4–7° C. before and during the time of ovulation had little effect upon the subsequent penetration of the eggs by spermatozoa. With a hypothermia of 0–1° C., however, there was almost complete and apparently permanent inhibition of spermatozoon entry. There seemed to be no reason to ascribe this effect to direct influence of cold on eggs or spermatozoa. Probably the failure of penetration arose largely from delay of ovulation, coupled perhaps with some derangement of the tubal environment.

Hypothermia after ovulation but before and during the time of spermatozoon entry into the eggs temporarily but completely inhibited penetration when the body temperature was kept below 33° C. The likely explanation appears to be that 'capacitation' is prevented or greatly slowed by such temperatures.

Hyperthermia, induced before and during the time of ovulation, somewhat reduced the mean number of eggs ovulated (from 10·5 to 7·5) and the proportion penetrated by spermatozoa (from 97 to 66%). The proportion of eggs penetrated by two or more spermatozoa was increased (from 19 to 28%), but the frequency of polyspermic fertilization did not change significantly.

Hyperthermia after ovulation but before and during the time of spermatozoon entry also led to a greater proportion of eggs containing two or more spermatozoa (31 %), but in addition it increased the incidence of polyspermy (from 2·5 to 21·2 %). Larger rats (170–200 g.) subjected to hyperthermia showed a higher frequency of polyspermy (34 %) than smaller rats (100–130 g.; 13 % polyspermy).

Evidently the processes involved in the 'zona reaction' are reduced in efficiency by hyperthermia whether applied before or after ovulation. On the other hand, the block to polyspermy depends on processes that are much more sensitive to heat after ovulation. It is considered that the slowing down of the block to polyspermy is associated with ageing of the egg, that the ageing is accelerated by hyperthermia and that this effect is promoted in older rats.

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EARLY REACTIONS OF THE RODENT EGG TO SPERMATOZOON PENETRATION

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INTRODUCTION

Evidence has been brought forward to show that the zona pellucida in the eggs of many species of mammals undergoes a change after the penetration of the first spermatozoon, as a result of which the penetrability of the zona to further spermatozoa is greatly reduced (Braden, Austin & David, 1954). Observations on the angular relations of the points of entry of spermatozoa through the zona pellucida in the rat suggested that the 'zona reaction' is initiated in the zona itself, at the point of entry of the first spermatozoon and is then propagated through the zona. Thus, the mammalian egg appeared to have two independent protective devices against polyspermy, namely, the zona reaction and the block to polyspermy in the surface of the vitellus. In some species (e.g. the sheep and dog) the zona reaction is highly developed; in others (e.g. the rabbit and mole) protection is vested chiefly, perhaps exclusively, in the vitelline block; in others again (e.g. the rat and mouse) both processes play a role in keeping out extra spermatozoa.

Recently, Rothschild (1956, and personal communication) suggested that the initial response to spermatozoon entry occurs in, and is propagated through, the surface of the vitellus. As a result, an agent is released from the cortex which passes across the perivitelline space and exerts a 'tanning' effect upon the zona. Rothschild pointed out that the release of substances, associated with a change propagated through the vitelline cortex, seems to be of wide incidence in lower animals. From this point of view there would not be two independent protective mechanisms against polyspermy in the mammalian egg, but instead a single response that could find expression in either the zona reaction or the block to polyspermy, or both, according to the species.

With the object of testing this hypothesis the process of spermatozoon penetration as it occurs *in vivo* has been further investigated. Some new data have been obtained and in this paper the attempt is made to give a logical account of the reactions shown by the egg to spermatozoon penetration. Direct evidence on the 'tanning' hypothesis is still lacking, but from indirect evidence it seems very likely that the block to polyspermy and the zona reaction are indeed manifestations of the same underlying response.

METHODS

Rat, mouse and hamster eggs were examined by the phase-contrast microscope, as in previous investigations, but on this occasion care was taken to study the eggs before, as well as after, compression under the cover-slip. It is much more difficult to discern details within uncompressed eggs, but a better idea is obtained of spatial relations of penetrating spermatozoa.

OBSERVATIONS

Details of spermatozoon penetration. Little can be said of the passage of the spermatozoon through the zona pellucida as it is very rare to find an egg with a spermatozoon head still in the thickness of the zona. The rarity of such a finding bears witness to the rapidity with which passage through the zona must occur. Generally the direction of passage is vertical to the surface or at a slight angle from the vertical.

In unpenetrated rat and mouse eggs the perivitelline space is smaller than in penetrated eggs, but is quite large in comparison to the size of the spermatozoon head. (This is even more evident in the hamster egg, in which the perivitelline space is unusually large—Austin, 1956*a*). The vitellus shows a distinct elevation at one point and beneath this lies the second maturation spindle. The perivitelline space is wider in the region around the elevation than it is elsewhere. On penetrating the zona the spermatozoon head projects into the perivitelline space and shortly makes contact with the surface of the vitellus (Fig. 1*a*). Clearly this will happen sooner if penetration occurs where the perivitelline space is narrow. If the spermatozoon should enter in the region near the vitelline elevation an appreciable length of the mid-piece may pass into the perivitelline space before the head makes contact with the vitellus. In this region, therefore, the point of contact with the vitellus may not be immediately below the site of penetration through the zona. The lack of coincidence will be further increased if the angle of penetration diverges much from the vertical.

Following contact, the spermatozoon head apparently adheres to the vitelline surface, since the residual motility shown by mid-piece and tail becomes translated into a reciprocating movement of the head along an arc centred at the point of contact. Initially, only an anterior part of the head touches the vitellus, but soon, with the passage of more of the mid-piece into the perivitelline space, the head comes to lie flat on the vitelline surface with the whole of the contiguous side attached to the vitellus (Fig. 1*b*). The reality of the attachment is evident from the close application of the head to the vitellus, from the obvious restriction of movement, and from the manner in which the vitelline granules near the head are disturbed when the tail moves. The appearance is quite different from that presented by supplementary spermatozoa in rat and mouse eggs which are free to move progressively through the perivitelline fluid.

The condition represented in Fig. 1*b* is a common finding; occasionally, however, spermatozoa are found in such a position that it is clear that entry into the vitellus

occurred with little change in the orientation of the head from that shown in Fig. 1*a*.

The next step involves the passage of the head through the vitelline surface so as to lie in the immediately subjacent cytoplasm (Fig. 1*c*). Only then do the earliest

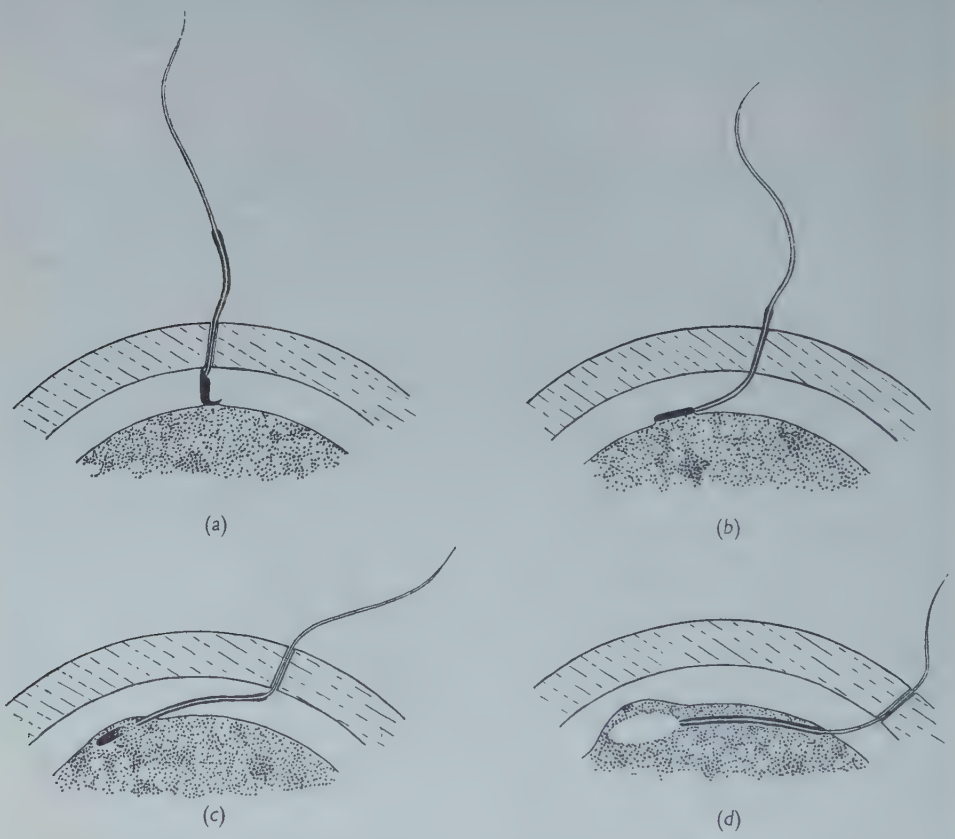

Fig. 1. The figures illustrate four stages in the penetration of a rodent egg by a spermatozoon. (*a*) The spermatozoon head has just passed through the zona pellucida and made contact with the vitellus. (*b*) The spermatozoon head is lying flat upon the vitelline surface to which it is now attached. (*c*) The whole of the spermatozoon mid-piece has entered the egg and the head has passed through the surface of the vitellus. The spermatozoon head shows an early phase in its transformation to a male pronucleus—the posterior end of the head is becoming indistinguishable from the egg cytoplasm. (*d*) The head and mid-piece of the spermatozoon have now entered the vitellus. Transformation of the head has proceeded to the stage immediately before the appearance of nucleoli. The cytoplasmic elevation over the spermatozoon head, just evident in (*c*) has now become much larger.

signs become evident of the metamorphosis of the head into a male pronucleus. (The structural changes in the development of pronuclei have been described in detail elsewhere—Austin, 1951*a*; 1956*a*). During the early part of pronucleus formation, the spermatozoon mid-piece often lies in the perivitelline space and the tail may still be projecting some distance outside the zona. More often, however, most of the spermatozoon has by now entered the vitellus (Fig. 1*d*). For the

spermatozoon to have achieved this position the vitellus must presumably have undergone some rotation, the motility of the mid-piece and tail supplying the motive force. After the head has passed through the surface of the vitellus a distinct elevation of the vitelline cytoplasm develops in this region (Fig. 1*c*, *d*); the elevation subsides as the head, and later the male pronucleus, moves towards the middle of the egg. This elevation may be analogous to the 'fertilization-cone' of invertebrate eggs.

Incidence of eggs with spermatozoon only in perivitelline space. Forty-seven hamsters were killed when the eggs were undergoing the early stages of fertilization. The animals yeilded 192 eggs that had been penetrated by spermatozoa but in which pronucleus formation had not yet occurred. In 34 eggs (18%) the spermatozoon was still in the perivitelline space. Seventeen of these eggs were closely studied and in all of them the head of the spermatozoon was found to be in contact with, and apparently attached to, the surface of the vitellus. Thirty eggs exhibited the metaphase of the second maturation meiosis, 1 egg showed the anaphase stage and in 3 eggs the second polar body had been abstricted.

Forty hamsters were killed at 2.0, 3.0, 4.0 and 5.0 p.m., ten at each hour. These animals had been kept under controlled illumination and it has been shown that, under such conditions, ovulation occurs principally between 12.0 noon and 4.0 p.m. (Austin, 1956*a*). A total of 304 eggs was recovered of which 176 had been penetrated by spermatozoa. In 21 (12%) of the penetrated eggs the spermatozoon was in the perivitelline space. The frequency of penetrated eggs increased from 2% at 2.0 p.m. to 78% at 5.0. p.m. From data of this kind an approximate estimate can be made of the mean time spent by the spermatozoon in the perivitelline space, assuming a constant increase in the rate of penetration over the test period (Austin & Braden, 1954*a*). The figure obtained in this instance is 28·4 min. or about half an hour.

In rats and mice, also, eggs that had spermatozoa only in the perivitelline space were carefully examined. In every one of 12 rat eggs and in 22 out of 25 mouse eggs the head of the spermatozoon was attached to the surface of the vitellus. The spermatozoa were quite free in the perivitelline space of the remaining 3 mouse eggs. The rat eggs were obtained after normal mating, but the mouse eggs were recovered from animals that had copulated after ovulation. Even after normal mating in mice it is not unusual to find an occasional egg with one or more spermatozoa in the perivitelline space and none in the vitellus. It is possible that some of the mouse eggs were inherently unfertilizable.

The incidence of polyspermy in hamsters. From ninety-six hamsters 927 eggs were recovered, of which 725 were undergoing fertilization or were in the 2-cell stage. In such eggs the diagnosis of polyspermy is reasonably certain. There were altogether 10 polyspermic eggs (1·6%), all of which were dispermic. In not one of the 725 penetrated eggs, however, was there a supplementary spermatozoon in the perivitelline space.

Effect of induced hyperthermia on spermatozoon penetration in the mouse egg. Mice were permitted to copulate after the time of ovulation and were then placed in a heated box maintained at 35–37° C. for 1–2 hr. On slaughter 6–8 hr. after mating,

nine mice yielded 81 eggs in which the zona pellucida had been penetrated by spermatozoa.

Of these eggs, 30 were found to be undergoing early parthenogenetic development similar to that previously described (Braden & Austin, 1954*b*), each having one to seven spermatozoa within the perivitelline space (mean 3·17) and none within the vitellus.

In a further 27 eggs parthenogenetic development had apparently begun, but this had not prevented the entry of a spermatozoon into the vitellus. The male pronucleus was represented by a small vacuole or a small atypical nucleus. Only 4 eggs had supplementary spermatozoa so that the mean number of spermatozoa penetrating the zona was 1·19 in this group.

The remaining 24 eggs had normal male and female pronuclei; 1 egg was dispermic and 2 eggs had one supplementary spermatozoon each. Thus, the mean number of penetrating spermatozoa was 1·13.

There is clearly a very significant difference between the first group and the second and third groups in the number of spermatozoa penetrating the zona pellucida. With one exception only, all three classes of eggs were found in each mouse.

DISCUSSION

The process of spermatozoon entry through the zona pellucida and into the vitellus, as observed in the present study, is essentially the same as that previously described for the rat egg (Austin, 1951*b*). As surmised then, the vitellus appears to play the more active role in the last phase of spermatozoon penetration, engulfing the spermatozoon much as an amoeba engulfs a food particle. In the earlier investigation, however, the eggs were always compressed between cover-slip and slide, and consequently it was not possible to say for certain whether the spermatozoon was free during its stay within the perivitelline space. This, then, constitutes the principal new observation, namely that, in rats, mice and hamsters, the spermatozoon head, on entering the perivitelline space, almost immediately makes contact with the surface of the vitellus and adheres to it. Here it stays for a mean period of about half an hour in the rat (Austin & Braden, 1954*a*), mouse (Braden & Austin, 1954*a*) and hamster (present data), before passing into the vitellus. Attachment is considered to represent a function rather of the vitelline cortex than of the spermatozoon head and to be a necessary preliminary to the absorption of the spermatozoon into the vitellus. Entry of the spermatozoon will, therefore, be precluded by abnormalities of either gamete, inherent or experimentally induced, that prevent this attachment. Experimental inhibition of spermatozoon attachment seems to have been achieved by Parkes, Rogers & Spensley (1954): they observed that unfertilized rabbit eggs, containing spermatozoa only in the perivitelline space, were more commonly found when the semen used for insemination was treated with certain enzyme inhibitors.

The prompt attachment of the spermatozoon head makes it possible for the vitellus to respond almost immediately after the penetration of the zona pellucida.

One of the earliest responses is the development of the block to polyspermy. Once this block has been established spermatozoon attachment can no longer occur—supplementary spermatozoa were always observed to be free in the perivitelline space. In the rat the block to polyspermy appears to be a specific reaction, for it was not found to be evoked by stimuli that were highly effective in inducing activation of the eggs (Austin & Braden, 1954*b*). The same may also be true for the mouse egg, although the evidence is somewhat equivocal: in the mice subjected to hyperthermia spermatozoa had penetrated into the vitellus of a number of activated eggs but there were other activated eggs in which all the spermatozoa remained free in the perivitelline space. It is possible that in the latter group the block to polyspermy had been evoked by the elevation of body temperature. However, a more likely explanation seems to be that the hyperthermia had deleteriously affected the vitellus, preventing it from forming an attachment with the spermatozoon head, since the efficiency of the block to polyspermy has been shown to be reduced after heat-shock treatment or hyperthermia in rats (Austin & Braden, 1954*b*, Austin, 1955, 1956*c*) and heat-shock treatment in mice (Braden & Austin, 1954*a*).

The hamster egg possesses an array of granules in the vitelline cortex that disappears during spermatozoon penetration (Austin, 1956*b*). The phenomenon is similar to that shown by sea-urchin eggs (Moser, 1939; Endo, 1952). In the hamster the cortical granules apparently disappear as soon as the spermatozoon head makes contact with the vitellus, but are unaffected by the spontaneous or artificial activation of the egg. These facts suggest that the disappearance of cortical granules is a visible manifestation of the block to polyspermy. Available data indicate, however, that this is not so: among 725 penetrated hamster eggs there were 10 polyspermic eggs but not a single instance of an egg with a supplementary spermatozoon in the perivitelline space. There is thus good reason to believe that the hamster egg does not develop a block to polyspermy. On the other hand, the same data testify to the efficiency of the zona reaction in the hamster egg and upon this the egg seems to depend exclusively for protection against polyspermy.

In contrast to hamster eggs, 21 % of rat eggs and 16 % of mouse eggs were found to have supplementary spermatozoa (Braden *et al.* 1954); in these species, therefore, the zona reaction is much slower and greater dependence is placed upon the block to polyspermy. In rats and mice, too, no evidence of a cortical granule response has been found. The hamster egg, then, has two very well-developed reactions, the cortical granule response and the zona reaction, and it is logical to suggest that they are casually related, the former leading to the latter. Such a relationship would be roughly analogous to that observed in sea-urchin eggs.

The reactions shown by the hamster egg are of particular interest, for they could well represent the visible signs of the operation of such a mechanism as that suggested by Rothschild (1956), namely, the propagated release by the vitelline cortex of a 'tanning' agent which passes out to reduce the penetrability of the zona to spermatozoa. The apparent absence of a cortical granule response from rat and mouse eggs does not necessarily preclude their having essentially the same mechanism, albeit in a less well-developed and less easily visible form. Available evidence,

indeed, favours the presence of such a mechanism. The eggs of all three species display the same attachment of spermatozoon to vitelline surface, and the stay of the spermatozoon in the perivitelline space is of about the same duration. Moreover, heat treatment, which affects the vitellus in the rat and mouse, increases the penetrability of the zona to spermatozoa (rat: Austin & Braden, 1954*b* and Austin, 1956*c*; mouse: Braden & Austin, 1954*a*). The present mouse data are particularly significant in this connexion, for it was only those eggs that contained no spermatozoon in the vitellus, and that presumably therefore had been rendered incapable of forming an attachment with the spermatozoon that had been penetrated by more spermatozoa than usual. Consequently, the failure of the zona reaction can be related to the induction of abnormality in the vitellus. Similar evidence is provided by the occasional eggs that contained exceptionally large numbers of spermatozoa: 4 rat eggs with between 15 and 60 spermatozoa each were reported by Braden *et al.* (1954), 1 rat egg with about 23 spermatozoa by Austin (1951*b*), 1 pocket-gopher egg with about 65 spermatozoa by Mossman & Hisaw (1940), and 1 mouse egg with 50 to 60 spermatozoa (unpublished data). In all these eggs the vitellus was clearly degenerate, and again failure of the zona reaction could be ascribed to abnormality of the vitellus which precluded spermatozoon attachment.

The observation that in rat eggs containing 2 spermatozoa the sites of entry through the zona are more frequently in the opposite than in the same hemisphere (Braden *et al.* 1954) is consistent with this hypothesis. Since the spermatozoon would generally make contact with the vitellus immediately after passing through the zona the release of the supposed 'tanning' agent would be likely to spread from the point of contact, and consequently the zona reaction would commence near the site of penetration and then progressively involve the rest of the zona.

The sequence of events in the final stages of spermatozoon penetration is therefore held to be as follows: on passing through the zona pellucida the spermatozoon head makes early contact with the vitellus and becomes attached to the surface. Attachment may initially involve only the anterior end of the spermatozoon head but more commonly the whole of one side of the head is closely applied to the vitellus. As a result, changes occur in the vitelline cortex, involving either the development of the block to polyspermy or the release of an agent that induces the zona reaction, or both, depending on the species. These two reactions appear to be elicitable only by the spermatozoon; they are presumably both propagated through the vitelline cortex; they may represent different manifestations of a single basic response. After the spermatozoon head has been attached to the vitelline surface for about half an hour it is absorbed by the vitellus, while the cortical cytoplasm becomes elevated at the site of absorption to form a projection similar to that above the second maturation spindle. Progressively, the mid-piece and tail of the spermatozoon pass into the vitelline cytoplasm. During the passage of the spermatozoon head through the outer regions of the vitellus its transformation into a male pronucleus begins; resumption of the second meiotic division of the egg—a non-specific response—occurs at about the same time.

SUMMARY

In the rat, mouse and hamster the spermatozoon passes rapidly through the thick, homogeneous zona pellucida surrounding the egg and the head almost immediately becomes attached to the surface of the inner cytoplasmic mass or vitellus. As a result of this attachment a block to polyspermy is developed in rat and mouse eggs. In the hamster a block is apparently not formed. It seems likely, therefore, that the disappearance of cortical granules in the hamster egg, also an outcome of contact with the spermatozoon head, could signal the release of an agent that is responsible, after crossing the perivitelline space, for bringing about the zona reaction, reducing the penetrability of the zona pellucida to spermatozoa. Data suggest that this mechanism exists also in rats and mice, although a cortical granule response has not been distinguished in these animals. Thus, attachment of the spermatozoon head to the vitellus probably elicits both the zona reaction and the block to polyspermy.

These changes appear to be specific to spermatozoon penetration and to be initiated before the spermatozoon head passes through the surface of the vitellus and before the resumption of the second meiosis.

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THE NERVES AND MUSCLES OF MEDUSAE

V. DOUBLE INNERVATION IN SCYPHOZOA

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INTRODUCTION

The first paper of this series (Horridge, 1954*b*) was a study of the through-conducting, unpolarized net of relatively large nerve cells which propagates a single nerve impulse over the whole bell of *Aurellia* at each beat, and by virtue of its symmetrical properties and relatively rapid conduction rate is responsible for the mechanically desirable symmetry of the contractions. This is the net, common to Rhizostomeae and Semaeostomeae, which was originally described by Shäfer (1878) in *Aurellia*, and which has provided a model for much discussion of the physiology and the phylogeny of the elementary nervous system. However, this nerve net is only a specialized part of the peripheral nervous system. Its action as a conducting system is confined to the excitation of the main muscles of the bell and it may be considered as equivalent, on functional and physiological grounds, to the motor giant fibre systems found in some other invertebrate groups. This limitation of the function explains the relatively straightforward physiological properties. In fact, under normal conditions, this whole net acts on the muscle as a single motor nerve.

In the literature one can find descriptions of several different kinds of other excitation which cross the subumbrellar epithelium without giving rise to a contraction wave *en route*. These fall into four groups. First, historically (Romanes, 1877), is the 'excitational wave' of tentacle retraction which crosses the bell of *Aurellia* at half the velocity of the contraction wave and initiates a beat from the first tentaculocyst that it meets. This is a special case of the indirect acceleration of the rhythm by a mechanical stimulation at a distance from the ganglion. Secondly, Bozler (1926*b*) has described the response of the oral arms of *Pelagia* to stimulation at the margin of the bell and also the outward propagation of slow waves of contraction across the circular muscle with a velocity of about 1 cm./sec. In confirmation and extension of Fränkel's (1925) work on *Cotylorhiza*, Bozler has also described the compensatory movement of *Cotylorhiza* (1926*a*) and *Pelagia* (1926*b*) in which, after displacement, an asymmetrical component added to the beat serves to bring the animal back to an even keel. Considering these different examples of conduction, Bozler came to the conclusion that each reaction had its basis in a separate nerve net. Thirdly, Horstmann (1934*a*, *b*), besides briefly describing the compensatory movement of *Cyanea*, noticed that for a short time

after removal of a marginal ganglion of *Aurellia* the neighbouring part of the margin remained bent inwards during the pauses between contractions. Following Fränkel, he inferred a regulatory effect of the ganglia upon the 'tonus' of the surrounding circular muscle, and assumed that this was also the mechanism of the compensatory movement. Finally, Mayer (1906), observing a contraction wave continuously circulating round a ring cut from the bell of *Cassiopea xamachana*, found that the amplitude of the contractions increased for a short time after a general mechanical disturbance, though the frequency of the contractions remained the same. This observation escaped the attention that it deserved from later workers on medusae, who confined themselves to the influence of the frequency upon the amplitude of the contraction.

In the ephyra larva of *Aurellia* it has been found (Horridge, 1955*c*) that the extra-ganglionic nervous system is divided into two nerve nets which meet and interact at the marginal ganglia. The first, named the diffuse nerve net, spreads over both oral and aboral surfaces. It contains primary sense cells and motor fibres, and is responsible for the contraction of the radial muscle of a single arm, together with the co-ordinated movement of the mouth in the feeding response. It is reminiscent of the oral disk conducting system which controls the tentacles and the feeding reaction in anemones (Pantin, 1935). The diffuse nerve net, besides having a local sensory and motor function, also acts on the rhythm of the marginal ganglia and inhibits the beat during the feeding response. The other nerve net is the giant fibre system, corresponding histologically and functionally with Shäfer's net in the adult *Aurellia*, but the anatomical arrangement is in accordance with the larval division of the muscles into radial and circular blocks. This through-conducting net is superimposed histologically and functionally on the diffuse nerve net, and it brings with it the rapid co-ordination essential for the swimming movement. In the adult *Aurellia* the feeding response is lost, but the diffuse nerve net persists as the subumbrellar conducting system, which is presumed to be responsible for Romanes's 'excitational continuity', i.e. to conduct the tentacular waves and to act indirectly on the rhythm.

In *Cyanea* there are two nerve nets which are histologically similar to those of the ephyra of *Aurellia*. Here the feeding response of the larva has again disappeared, but the asymmetry of the compensatory movement makes it likely that double innervation has persisted into the adult in muscles sufficiently powerful to give a record.

For *Cassiopea* (Rhizostomeae), there is no histological evidence available, but in the related *Rhizostoma* (Bozler, 1927) there is a network of large bipolar cells corresponding to the giant fibre net of semaeostomes, and also a network of smaller multipolar cells that corresponds with the diffuse net. Mayer's observation, quoted above, requires some extension of the simple all-or-nothing action of the giant fibre net, and it will be shown that the relevant reactions of *Cassiopea* are closely similar to those of *Cyanea*. Also in *Nausithöe* (Coronatae) it is now found that the responses can only be explained in terms of two overlying nerve nets which interact at the marginal ganglia, and these two nets are similar to those

of the other orders. In these three orders, at least, there is emerging a common plan which includes double innervation of some muscles where an asymmetrical movement of the bell is mechanically necessary for feeding or for the compensatory movement.

MATERIAL AND METHODS

Large specimens of *Cyanea* were obtainable at Millport in July 1955. They were of the red variety and usually 20–30 cm. diameter. The specimens of *Cassiopea* were studied at Ghardaqa, Red Sea, in September 1955. The *Nausithöe* appeared at Naples in March 1952, and the results there obtained could not be interpreted until recently when they were found to be closely comparable with the results obtained using the ephyra of *Aurellia*.

The methods employed have been of the simplest kind, electrical stimulation by condenser discharges and recording by isotonic levers on a smoked drum. Fresh material was found to be essential.

A. SEMAEOSTOMEAE. *CYANEA CAPILLATA* ESCHSCHOLTZ

The compensatory movement of Cyanea

The specimen of *Cyanea* is held immersed in a tank of sea water with the main axis horizontal, and maintained in this position by two small hooks through the exumbrellar part of the jelly. The support is arranged so that the bell can be rotated and any segment brought uppermost. In this position the spontaneous

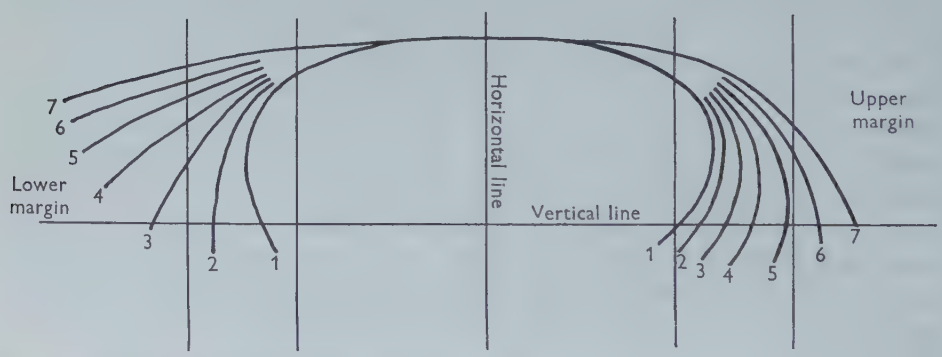

Fig. 1. Diagrammatic representation of successive stages, 1–7, in the relaxation of a bell of *Cyanea* which is mounted on its side. The uppermost margin, on the right, contracts to a greater extent, relaxes slower and less completely than the lower margin. The figure is turned on its side for convenience.

beats are consistently asymmetrical. At each beat the uppermost part of the margin contracts to a greater extent, remains in a contracted state for a longer time than the lower side, and also fails to relax as completely before the next beat. A representation of successive positions is shown (Fig. 1). The bell may be rotated about its axis so that any segment is brought uppermost, but this does not affect

the orientation of the asymmetry, which remains determined by its relation to the vertical. In freely swimming animals it is readily observed that the mechanical effect of this asymmetry is to bring a tilted animal back to an even keel, exumbrellar surface upwards.

After removal of seven of the eight tentaculocysts the asymmetrical character of the contractions persists as long as the remaining tentaculocyst is in the uppermost position, but now on rotating the bell the asymmetry is lost. Furthermore, the average rate of beating is greatest when the single tentaculocyst is in the uppermost position, and the spontaneous rhythm may stop altogether when the bell is rotated to bring the tentaculocyst to the lowest position. This agrees with another observation, that in tilted normal animals the contraction wave is often initiated at the uppermost marginal ganglion.

Confirming Fränkel (1925), these observations indicate that the local effect which forms the asymmetrical component is to be found at the uppermost margin in the compensatory movement. The mechanism that is important for the life of the whole animal is one for locally retarding the process of relaxation.

The slow waves

When a small specimen of *Cyanea* (5–10 cm. diameter) is spread out upside down in a dish of sea water it can be seen that relatively slow waves of contraction spread across all the muscles with intervals of $\frac{1}{2}$–2 min. between successive waves. In their most characteristic form these waves begin at the inner edge of the circular muscle and spread outwards. Under a binocular microscope each wave can be seen as a regular sequence of contractions of the individual folds into which the muscle sheet is thrown. At any point the contraction lasts 2–4 sec. as in the normal contraction wave of the beat. Having traversed the circular muscle, the slow wave appears in the radial muscle, which means that excitation crosses the intervening muscle-free area. The radial muscle contracts largely as one unit with a relatively slow response that may last 15–120 sec. As recorded by writing levers this response of the radial muscle is of smaller amplitude than in the normal contraction wave.

In a small specimen these outwardly moving waves often succeed each other in a regular sequence, or, if the animal is quiescent, they may be provoked by a gentle movement or pinch of the oral filaments. At times spontaneous waves cross the bell from side to side, and in response to a small prod or pinch of a marginal lappet a slow wave may be initiated, first in the appropriate radial muscle, then followed by a centripetal wave across all or part of the circular muscle. Two waves travelling in opposite directions cancel out where they meet. Across the circular muscle fibres the velocity of the wave is 0·15–0·25 cm./sec., but along the line of the fibres it is about 2 cm./sec. However, here it must be borne in mind that the length of the pathway across the fibres is about ten times the apparent length on account of the deep folds of the muscle sheet. In the radial muscle a simple velocity could not be measured. Observed under a binocular

microscope the sustained, weak contraction of the whole muscle appears to be a combination of many irregular local contractions out of phase with one another.

In large specimens of *Cyanea* (up to 35 cm. diameter) the spontaneous slow waves are also found (Fig. 2). A contraction typical of the slow wave may follow a local mechanical stimulation, but the response is rarely propagated to the next muscle block. It is only in the radial muscle preparations, described below, that slow contractions regularly follow stimulation of a nearby muscle-free area.

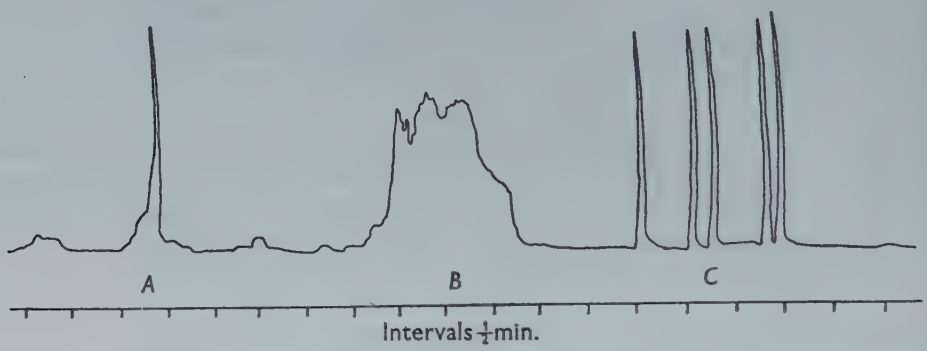

Fig. 2. Spontaneous responses of a radial muscle, showing the contrast between the record of a slow wave *B* and a series of normal beats *C*. At *A* is a small slow contraction terminated by a beat which it has initiated. Figs. 2 and 4–7 are traced from smoked drum records.

Action of the diffuse net on the marginal ganglia

The bell of a small specimen of *Cyanea* is pinned to wax on the bottom of a dish of sea water in such a way that the bell can beat freely, but with one of the tentaculocysts held firmly by pins through the jelly so that it can not tilt or vibrate. All the other tentaculocysts are removed and the preparation left for some hours to recover from the shock of the operation. It is consistently found that a gentle mechanical stimulation, such as rubbing the subumbrellar surface with a straw, is followed by the acceleration of the rhythm of the distant tentaculocyst. The excitation can spread across the whole bell. It is sometimes, but not necessarily, accompanied by a typical slow wave of contraction. A more severe stimulation, particularly within 3 cm. of the tentaculocyst is often followed by a long pause. Some preparations, especially those with the tentaculocyst upside down, show no acceleration but only the long pause. Comparable results, showing both acceleration and inhibition of the rhythm following distant stimulation, are found if the same experiment is repeated with *Aurellia*, but here there are no slow waves; instead, the spread of excitation in the diffuse nerve net is marked in some specimens by the contraction of the marginal tentacles. As a special case of the acceleration of the ganglion some preparations give, after a few seconds delay, a single beat in response to a single mechanical stimulation of the diffuse nerve net. For *Aurellia* this observation is described in detail by Romanes (1877); in *Cyanea* examples occur in the records shown.

The responses of the radial muscle

A pair of radial muscles, with a muscle-free area and tentaculocyst between them, is cut from the bell of a large specimen of *Cyanea*. Most of the jelly is carefully removed from the back of the muscles and the segment is pinned to wax under sea water as shown (Fig. 3). Two small hooks attached to writing levers are set in the jelly behind the remnants of circular muscle. The levers are loaded with

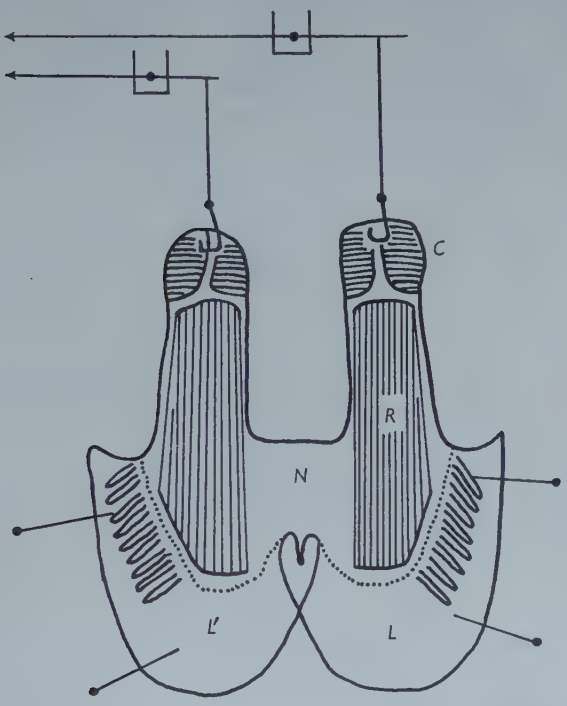

Fig. 3. The preparation of a pair of radial muscles of *Cyanea* with a tentaculocyst and muscle-free epithelium *N*. The dotted line shows the approximate lower limit of the giant fibre net. The italic capital letters indicate the alternative positions of the stimulating electrode; *N*, the giant fibre net; *L*, *L'*, the lappets; *C*, the circular muscle; *R*, the radial muscle.

a few grams to maintain a small tension in the muscles. To achieve satisfactory results it is necessary to use the lightest possible system with minimum friction on the drum. Stimulation throughout was with a fine silver wire, insulated to its tip, excited by a simple neon oscillator at 5 shocks/sec. The method of stimulating the lappet is important. The stimulator probe is moved gently along the lappet margin and inwards towards the limit of the radial muscle and is removed the moment any movement begins. Unless the contrary is stated, the muscle is not stimulated directly. The sensitivity of the lappet varies greatly from point to point, but an effort has been made to use minimal strength stimuli.

A single radial muscle gives a variety of responses, the characteristics of which

depend largely on the position of the stimulus. In some preparations, e.g. Fig. 4A, the height of the contraction is almost constant. In this record the first contraction at no. 1 is a spontaneous beat after a pause. It is followed at no. 2 by a localized contraction as a result of stimulation on the lappet, i.e. at *L* in Fig. 3. This is immediately followed at no. 3 by a 'spontaneous' beat which is initiated from the marginal ganglion as excitation in the diffuse nerve net arrives there from the lappet. The stimulus was then applied to the remnant of the circular muscle, i.e. at *C* in Fig. 3, and the response was normal. This trio is repeated at nos. 7–9; it

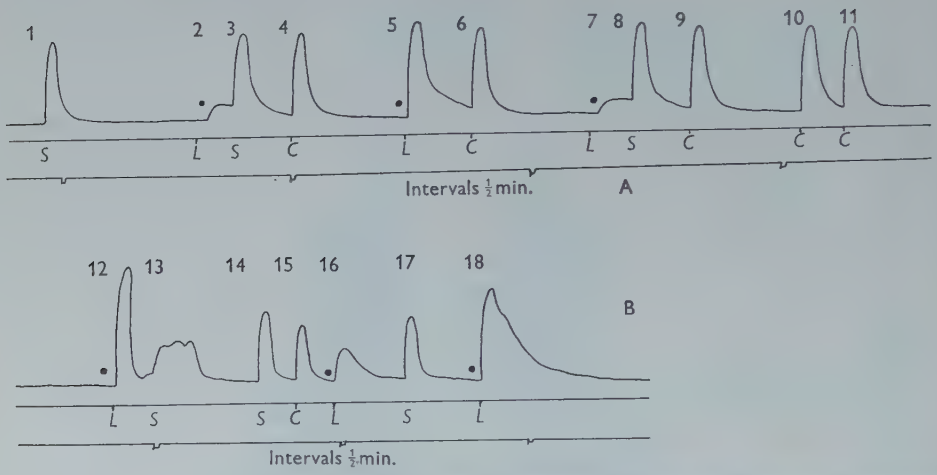

Fig. 4. Various responses of a single radial muscle as described in the text. The letters indicate the origin of the excitation: *S*, spontaneous beat; *L*, stimulus on lappet; *C*, stimulus on circular muscle.

was also attempted at nos. 5 and 6, and here stimulation at *L* produced a contraction wave directly, although the contraction no. 5 is drawn out by a slower relaxation. The shape of the contraction at no. 1, and especially no. 10, discount the possibility that the slower relaxation of no. 5 is explained by the lack of a previous contraction, i.e. to facilitation of relaxation.

A record showing a greater variety of forms of contraction is given in Fig. 4B. Here the first stimulus on the lappet is too close to the muscle and excites the giant fibre system directly at no. 12, but it also initiates a slow contraction at no. 13. Similar stimulation later produces at no. 18 a contraction where slow and fast waves are combined and at no. 16 a small slow wave is produced. The normal contraction waves at nos. 14, 15 and 17 again set a standard for comparison.

A difference in strength of the contraction waves is also noticeable in Fig. 4B. This effect is better shown in Fig. 5, from a different preparation. Here there is a large contraction with rapid relaxation at nos. 1 and 16–18, following stimulation of the giant fibres at *N* (Fig. 3), but smaller contractions follow stimulation of the circular muscle remnants at *C*. A slow contraction alone occurs at no. 15 and a lengthening of the quick contraction at nos. 10 and 12, possibly also at nos. 2 and 6.

An essential difference between the fast contraction and its slow component lies in the contrast between the through-conducting, all-or-nothing character of the contraction wave and the local, more variable character of the other. This is shown in Fig. 6 by a record of two neighbouring radial muscles *T* and *B*. Except for the symmetrical contraction, no. 4, which follows stimulation of the giant

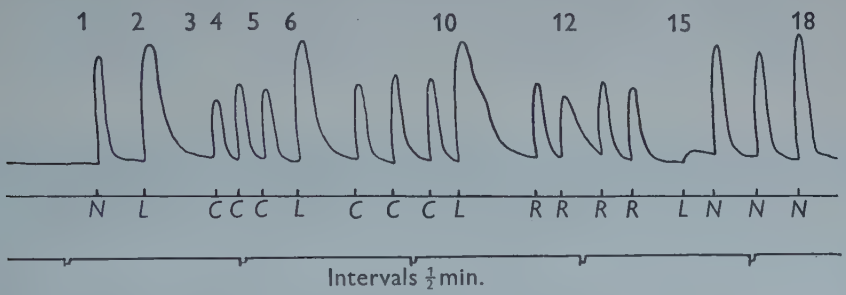

Fig. 5. The influence of the position of the stimulus on the height of the contraction. The letters indicate the point of stimulation: *N*, on the giant fibre net; *L*, on lappet; *C*, on circular muscle; *R*, on radial muscle.

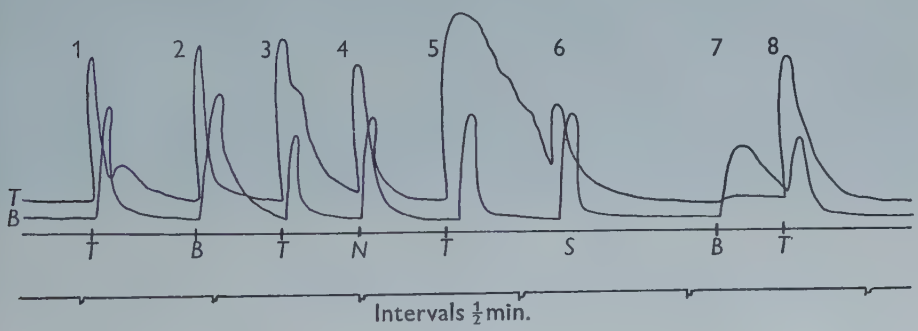

Fig. 6. Simultaneous records from a pair of radial muscles showing that the delayed relaxation is varied and local. The letter *T* indicates when the stimulus was applied to the lappet corresponding to the top trace, and similarly *B* for the bottom trace. The symmetrical contraction at *N* followed stimulation on the nerve net and *S* was a spontaneous beat.

fibre net at *N* (Fig. 3), the stimuli are applied at the points *L* or *L'* (Fig. 3). It is important to point out that in Fig. 6 the stimulus was never directly applied to the muscle. In the record a local effect is superimposed on the contraction wave and this effect here takes the form of an extension of the relaxation process. The muscle *T* shows the effect better than *B*, especially at no. 5, but the muscle *B* delays its relaxation at no. 2 and shows only a slow contraction at no. 7.

Other asymmetrical movements of Cyanea

In Fig. 7 is a recording from radial muscles on opposite sides of the bell of a large *Cyanea*. The upper trace intermittently shows abnormally great relaxations, but no importance can be attached to the contrast with the relative stability of the

lower trace, for this may be due to the recording system. However, where changes of the contraction height or of the base-line occur in the lower trace they appear to be independent of the changes in the upper trace. This can be interpreted in terms of the varied responses of the radial muscles already described, if we allow a little spontaneous or extraneous excitation in the diffuse nerve net.

There is, however, an entirely artificial type of asymmetrical contraction of the bell which may be produced by electrical stimulation at certain frequencies. The refractory period of the radial muscle of *Cyanea*, measured by Bullock's (1943) method is 1·1–1·3 sec. at 16° C. If the interval between electrical stimuli is

Fig. 7. Contractions of a pair of opposite radial muscles during a long series of spontaneous beats of an isolated segment of *Cyanea*. The difference in character of the traces may be partly due to the recording system, but this does not hide the disparity between the variations of the two sides. Read from left to right with contractions upwards.

adjusted to coincide with the refractory period of the muscle, or a simple fraction of it, asymmetrical contractions are readily recorded (Fig. 8). Presumably this happens because the muscles in various parts of the bell do not have identical refractory periods. This kind of experiment may be refined so that alternate large and small contractions are produced, as in parts of Fig. 8. The interpretation (Bethe, 1937) that such results disprove the all-or-nothing character of the nerve impulses is not valid. They are not concerned with the nerve impulse, but with the strength of the contraction. The alternative suggestion that the muscle consists of a population of units with a distribution of refractory periods gives a more satisfactory explanation.

The actions of the two nerve nets on the muscle appear to be independent, even when simultaneous. By comparison of muscles on opposite sides of the bell when first one muscle then the other is given a series of electrical stimuli, I have tried to find in *Cyanea* a local effect of the diffuse net on the neuromuscular facilitation of the giant fibre net. However, these experiments have always led to

a negative result as far as facilitation is concerned, though the preparations show the other effects described. This is in contrast to the situation to be described in *Cassiopea*.

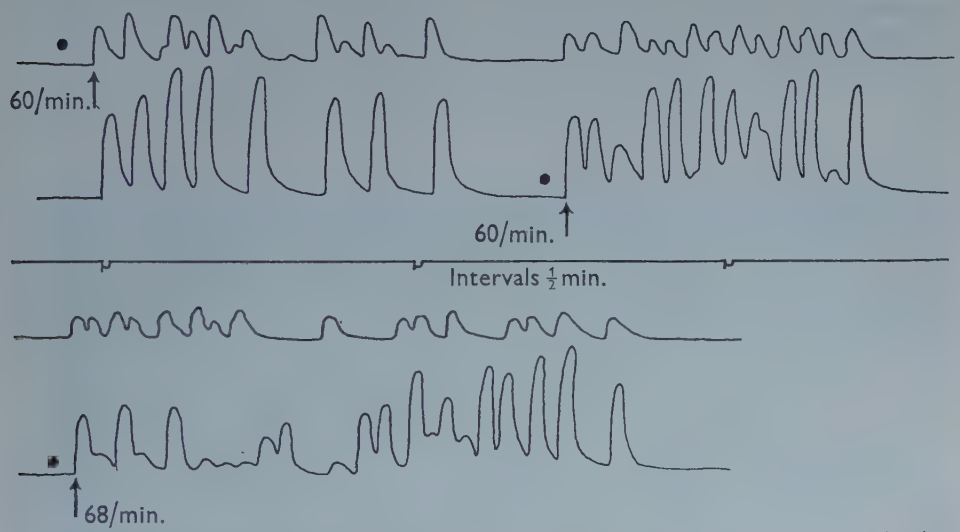

Fig. 8. Simultaneous records of a pair of neighbouring radial muscles of *Cyanea* when stimulated at the given frequencies, which are arranged to clash with the average refractory period of the muscle fibres. This is quite an artificial state of affairs. Stimulation is applied to first one muscle then the other, as indicated by the black spot.

B. RHIZOSTOMEAE. *CASSIOPEA ANDROMEDA* ESCHSCHOLTZ

The compensatory movement of Cassiopea

The specimen of *Cassiopea* is held on its side by hooks passing through the jelly of the exumbrellar surface. Under these conditions the asymmetry of the beat closely resembles that of *Cyanea* when similarly treated, although the arrangement of the effective muscles is quite different. The wave at each beat originates at the uppermost margin, which after each contraction remains more bent inwards than the lower margin and consequently has a smaller amplitude. However, after about half an hour in this position the pulsations and their origin often become quite symmetrical. When left still longer some specimens even reverse the initial asymmetry of the compensatory movement, which now tends to turn the animals upside down. These observations agree with the behaviour of freely swimming individuals, which turn to swim upwards when first disturbed, but which later turn upside down and settle on the bottom.

The slow wave of Cassiopea

A single electrical stimulus applied anywhere on the subumbrellar sheet of muscle fibres produces a normal contraction wave, but within the narrow clear band round the margin, outside the area of the circular muscle, the effect of a

stimulus is quite different. The neighbouring lappets, up to a distance of 10 cm., are drawn together by a local contraction of the circular muscle. With successive stimuli at intervals of 10 sec. this excitation spreads farther and in one case, for example, reached all round the bell at the third stimulus. The velocity is about 8 cm./sec. at 25° C. compared with 40–50 cm./sec. for the contraction wave. Under a binocular microscope the movement appears to be a contraction of the same fibres that contract at each beat, and subjectively it appears to occupy at any one place the same contraction time as the faster wave, but the slow wave only affects the circular muscle. If stimulation is continuous, the slow wave becomes a maintained contraction, but in *Cassiopea* this applies also to the contraction wave, and in each case the contraction of any small group of fibres comes in jerks, though the units are out of phase. A cut inwards from the margin through only the circular muscle has no effect on either of the two waves, and the cut is continued inwards without effect until only a narrow bridge is left in the radial muscle sheet.

Simultaneous slow and fast contraction in Cassiopea

A continuously circulating wave of contraction is maintained indefinitely once it is initiated in a large ring cut from the bell. The frequency of the contractions depends only on the velocity of the wave and the length of the closed circuit. The amplitude of the contractions soon settles down to a constant value as measured by the movement of an isotonic lever. Mayer (1906) has already found that when such a preparation is taken out of the water and thrown back, the amplitude but not the frequency increases. This experiment may be refined by use of electrical stimuli, but the result is effectively the same (Fig. 9). However, it can now be seen that besides the contraction wave circulating with a velocity of 40 cm./sec., the additional effect is an elevation of the line of peaks and usually also of the base-line. The second effect is propagated with a velocity of 8–10 cm./sec. and it lasts for more than one cycle of the contraction wave. These facts by themselves require for their explanation at least two independent pathways for the operation of any possible mechanism. In addition, the stimuli are equally effective even if applied outside the area where an electrical stimulus produces a contraction wave.

The question of what is happening at the point where the two nerve nets converge on the muscle fibres can hardly be touched for lack of data. The strength of a contraction depends on the activity of the diffuse net and on the interval since the previous contraction, but at present it is impossible to separate the effects of these two factors.

The value of the circulating ring preparation lies in the fact that here a contraction wave is available without any accompanying excitation in the diffuse nerve net. Normally an electrical stimulus produces an effect in both systems, but in the ring the effect is maintained in the through-conducting system, while in the diffuse nerve net it dies out. This shows an important difference between the two nerve nets, and makes possible the demonstration of the interaction of the two waves as they affect one group of muscle fibres.

C. ORDER CORONATAE. *NAUSITHÖE PUNCTATA* KÖLLIKER

The behaviour of the adult *Nausithöe* is closely similar to that of the ephyra larva of *Aurellia*. They have a similar habitat in the surface water with continual active swimming and a macrophagous feeding method. Both have two distinct systems of co-ordination over the subumbrellar surface, and it is sufficient to set out briefly the reactions of *Nausithöe*.

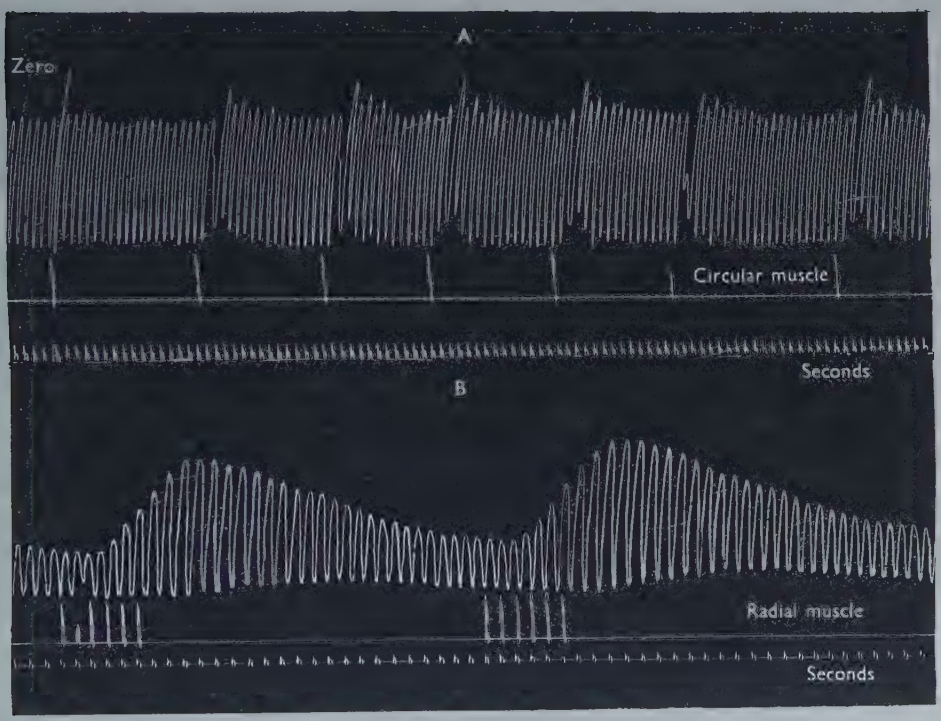

Fig. 9. *Cassiopea*. The responses of a ring which carries a continuously circulating contraction wave. A, top; single electrical stimuli applied 18 cm. from the recording point produce both a momentary dislocation of the circulating wave and also a shortening of the muscle, at both contraction and relaxation, for several cycles. B, five electrical stimuli at 1 sec. intervals produce a long-lasting effect on the height of the contraction, but here do not disturb the circulation of the wave. Both the radial and the circular muscle give the above reactions.

(1) The beat of the bell. It can be shown, by making incisions in different directions, that the nerve net responsible for the spread of the contraction wave is diffusely spread over much of the subumbrellar surface, as in other Scyphozoa. Artificial stimulation with a single shock elicits a single contraction of both the circular and radial muscles and it is likely, by comparison with other forms studied, that there is a network of through-conducting motor nerves which are responsible only for the symmetrical beat, and that one nerve impulse through the net is sufficient.

(2) The feeding responses may be summarized as follows:

A. A swing inwards towards the mouth of one or more of the relatively large marginal tentacles. These usually act independently of one another.

B. An inward bend of one or more of the marginal lappets by a maintained local contraction of their radial muscle.

C. A symmetrical retraction of the four lobes round the mouth, opening it wide by a maintained contraction of the whole sheet of radial muscle which is round the mouth.

The different responses above sometimes occur separately in response to food or mechanical stimulation, but normally they are co-ordinated together in such a way that captured food is passed to the mouth from the tentacle or lappet that receives it. Strong or continual stimulation of the subumbrellar surface is followed by an exaggerated feeding response that affects the whole animal, which promptly folds into a spasm lasting for several seconds.

(3) The rate of the rhythmical discharge of the marginal ganglia is slowed by a touch to the subumbrellar surface, and there is a temporary complete inhibition while the feeding reaction is completed.

Apart from differences in the details of the feeding responses the pattern is exactly the same as in the ephyra larva of *Aurellia*, and there is no doubt that two independent nerve nets are present, corresponding to those of semaeostomes. The principal point at present is the simultaneous existence of two types of contraction of the radial muscle of the lappets which, as in the ephyra, may be local and maintained or symmetrical and temporary. This can only be explained in terms of double innervation or by two types of muscle fibre and the latter is unlikely.

THE DIFFUSE NERVE NET

The term 'diffuse nerve net' is used here to cover all the peripheral nervous system except the through-conducting giant fibre net of the contraction wave. At present it appears to be one interconnected system, but this may later prove to be an oversimplification. In its histological appearance in *Cyanea* and *Nausithöe* it is similar to the diffuse net described in the ephyra larva of *Aurellia* (Horridge, 1955) and the two are closely comparable both physiologically and functionally.

The fibres of the diffuse nerve net of *Cyanea* are very thin with many bends, and often appear continuous from cell to cell. This should not be taken as evidence of continuity because the giant fibres, which are known to have contiguity synapses, appear as a continuous net when over-stained with methylene blue. The cell bodies, 6–10μ long, are smaller and rounder than those of the giant fibre net and are frequently multipolar. They branch and interconnect to form a fine network that ramifies everywhere among the epithelial cells. From the margin longer fibres run radially towards the ganglion and towards the radial muscle. The diffuse net also connects with the typical bipolar primary sense cells, each of which has a process that extends into the surface of the epithelium. These sensory cells are particularly abundant round the margin of the lappet, and

towards the marginal ganglion they merge into the columnar epithelium of ectodermal sensory cells on and round the tentaculocyst. In the marginal lappet, where the diffuse net is most easily stained, the giant fibres are absent both histologically and physiologically, and stimulation here produces a beat only indirectly or a slow wave in the nearby muscle. It is now realized that large fibres on the marginal lappet in an earlier diagram of *Cyanea* (Horridge, 1954*a*, fig. 2, p. 88) are part of the diffuse nerve net. The outer limit of the giant fibre net, as now determined by stimulation, is shown in Fig. 3 with a dotted line. The stimulation technique in the present experiments was intended to excite first the diffuse net and then follow with a contraction wave as the electrode moved into the area of the giant fibre net. The nature of the excitation in the diffuse net is unknown.

The histological appearance of the diffuse net of *Cassiopea* is not known, but physiologically it is very similar to that of *Cyanea*. It extends over the whole subumbrellar surface, including the oral arms. If the mass of the oral arms is removed and placed in sea water, aboral side upwards, it relaxes after an hour or so. A single electrical stimulus at any point initiates a wave of retraction of all the arms, which shorten by 2–3 cm. in 7–9 cm., and there is a slow bending of the stimulated arm and its branches.* In the quiescent animal this wave of excitation in the diffuse net initiates a series of beats from the marginal ganglia, but conversely it may slow the rhythm of a beating animal although the amplitude is at the same time increased.

On the other hand, the diffuse net of *Nausithöe* resembles that of the ephyra of *Aurellia* histologically, physiologically and in its anatomical plan. First there are primary bipolar sense cells, abundant in the tentacles and lappets of the margin. Connected to these is a fine net of bipolar and multipolar cells which is regionally differentiated. Round the mouth the fibres run principally in a circular direction over the inner sheet of radial muscle, but towards the marginal lappets long fibres run radially, which is in agreement with the co-ordination of one lappet and the whole mouth during the feeding response.

The network of multipolar cells and thin fibres of the diffuse net, stains with methylene blue in all Scyphozoa so studied, more easily in small specimens. The nerve cells in the tentacles and frills of the mouth are histologically similar, and are physiologically part of this net. When in company with the giant fibre net in the epithelium, it looks very similar to the net of multipolar cells coloured blue among the giant fibres in Bozler's (1927) figures. My own brief experience in staining *Rhizostoma* with methylene blue has convinced me that the diffuse net of semaeostomes is also present in *Rhizostoma* and is the net of multipolar cells that Bozler figures. In fact the diffuse net seems to be, histologically, physiologically and functionally, the generalized combined sensory-and-motor ectodermal net described in most groups of Coelenterates. As for the giant fibre system, Bozler (1927) divides his bipolar cells into three size groups, but I find no justification for separating these physiologically and consider them as all part of one and the same net.

* This observation reduces the value of the arm length as a specific character.

Absence of double innervation in Aurellia aurita

In *Aurellia* the conspicuous action of the diffuse nerve net in the feeding behaviour of the ephyra is lost at metamorphosis, and the evidence for any form of double innervation in the adult is very slender. There is Horstmann's (1934) observation that removal of a marginal ganglion is temporarily followed by a locally increased curvature of the bell, and his 'tonus' can be replaced by an explanation in terms of the two nerve nets, i.e. that local excitation of the diffuse net gives a local maintained contraction; but this is not supported by experimental evidence, and an explanation in terms of stress relief following the osmotic disturbances of the operation is equally tenable. The experiments that demonstrate double innervation in other species give negative results with *Aurellia*. No asymmetrical component has been found in the contraction of tilted animals and no slow waves across the bell have been described. In his study of facilitation in medusae, Bullock (1943) analysed the relation between the amplitude of contractions and the intervening intervals and he found the same relation satisfactory for both spontaneous and electrically initiated movements. However, by comparison with *Cyanea* and *Cassiopea* it is clear that this result applies only to those species where double innervation has a negligible effect, and of this *Aurellia* is the only known example.

CONCLUSIONS

The all-or-nothing character of the impulse at each beat is compatible with the consistent asymmetry of the compensatory movement if there is a separate local effect superimposed on the total effect of the giant fibre system. The local effect is the slow contraction in the muscle initiated (and perhaps there propagated) by the diffuse nerve net. The two responses may occur separately or simultaneously in one muscle. When they occur together the local effect in *Cyanea* takes the form of a lengthening of the relaxations; in *Cassiopea* the amplitude is increased.

Having before us the mechanism of the two nerve nets there still remains a problem to explain the compensatory movement when the animal is tilted. The asymmetrical component is consistently localized during a long succession of beats, but the diffuse net may in *Cyanea*, and usually in *Cassiopea*, conduct excitation across the whole bell. What is required is a mechanism which limits the area in which the double innervation is effective. This is possible if two impulses are initiated together from the upper ganglion and spread out, one in each independent net. However, because of the disparity in the two velocities it will only be in an area round the ganglion of origin that the excitation in the diffuse net will find muscle fibres that are not refractory. This suggestion has not been tested by experiment, but it brings together all known observations.

On a histological level there is no evidence of two types of muscle fibre, and on a large scale the muscles appear to be homogeneous and capable of both responses in every part. The only conclusion is that a double innervation is present. The innervating nerves here have the form of nets, which can be histologically

distinguished and separately stimulated. However, there is no histological picture of the nerve endings in the muscle. There is also the possibility, which has not been eliminated, that over short distances the propagation of the slow waves is from muscle fibre to muscle fibre, although the contraction is initiated by the diffuse net. It is therefore impossible to discuss in detail the innervation of the muscle fibres by both nerve nets.

Looking more broadly at the known examples of double innervation in Coelenterates, it is seen that they correspond with the functional requirement that a muscle must give a symmetrical and also a local response. It is an essential part of the organization of the ephyra larva that the feeding response is a maintained contraction of a single arm, but the swimming movement momentarily involves all arms. This is reflected in the co-ordination by two nerve nets. In the adult *Cyanea* and *Cassiopea* the double innervation persists and co-ordinates the compensatory movement. In *Nausithöe* the functional requirement and the corresponding pattern of the nervous system are closely similar to those of the *Aurellia* ephyra. However, in the adult *Aurellia* the diffuse net no longer acts on the muscle but only on the tentacles and the marginal ganglia. The existence of two separate but overlying conducting systems is established in the Hydromedusae (Horridge, 1955*a*, *b*), where, as in the ephyra, there is one system which co-ordinates the asymmetrical feeding responses and one which co-ordinates the beat. But in Hydromedusae the corresponding functional division into radial and circular muscles is an arrangement which does not require the double innervation of one group of muscles. By contrast with medusae of all kinds the co-ordination of most polyps is effected by a single net which is regionally differentiated. There is no indication of overlying independent systems or double innervation in most anemones. A description of two exceptions, the rhythmical Xeniidae and the swimming Boloceroididae, is in preparation.

The point of interaction of the two nerve nets in medusae is of especial interest. In semaeostomes the rhythmical centres of the beat are within the giant fibre system (Pantin & Vianna Dias, 1952), and presumably all other medusae have this pattern. These rhythmical nerve cells are strongly influenced by excitation in the diffuse nerve net, but the impulse at each beat is not propagated in the reverse direction, into the diffuse net. This is evidence that the diffuse net acts at polarized junctions where it accelerates—or, with too much stimulation, inhibits—the rhythmical nervous centres. The situation is exactly paralleled in the Coronatae and Hydromedusae, where in all studied forms except the Geryonidae, the principal effect is an inhibition of the rhythm when the radial system is active. The existence of two overlying conducting systems and a polarized interaction between them seems to be functionally necessary for the simultaneous but independent co-ordination of two activities, and is an essential concomitant of the bell-shaped form of a freely swimming medusa.

SUMMARY

1. The compensatory movements of *Cyanea* (Semaeostomeae) and *Cassiopea* (Rhizostomeae) are described. The asymmetry of these responses is not compatible with the properties of the through-conducting giant fibre net which propagates the contraction wave.

2. Stimulation experiments with *Cyanea* show that a second nerve net, called the diffuse net, acts locally on the muscles of the bell and delays the process of relaxation.

3. In *Cassiopea*, the responses of a ring of tissue containing a continuously circulating contraction wave are also evidence of a double innervation.

4. The responses of *Nausithöe* (Coronatae) are described and are interpreted in a similar manner.

5. In all three orders the two nerve nets are in places superimposed but are independent except where they meet at the marginal ganglia. Here the diffuse net acts irreversibly on the rhythmical units, which are known to be within the giant fibre net.

This work was carried out during a tenure of a senior award from the Commissioners for the Exhibition of 1851. I should like to thank the members of the staffs of the marine laboratories of Ghardaqa, Millport and Naples, who have allowed me to make use of their facilities and who have left me with pleasant memories of my visits.

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NITROGENOUS EXCRETION BY EMBRYOS OF THE VIVIPAROUS SNAKE *THAMNOPHIS S. SIRTALIS* (L.)

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Functional considerations of the placenta of saurian reptiles are limited virtually to assumptions based on known embryonic requirements and structural placental features (Weekes, 1935). It appeared that the nature of embryonic excretion might profitably be investigated in the garter snake whose young are born alive. The extreme solubility of urea, and the fact that the black snake embryo excretes only 20% of its nitrogenous waste as uric acid (Clark, 1953), suggest the possibility that embryos of a placental saurian reptile might pass urea and ammonium salts to the blood stream of the mother; whether some of the soluble nitrogenous excreta are eliminated through the placenta or whether all nitrogenous excreta are retained in the foetus and adnexa, the degree to which the embryo is dependent upon uric acid is of interest. Accordingly, assays of urea, ammonia and uric acid in the developing embryo were undertaken.

MATERIALS AND METHODS

Urea and ammonia were measured by the technique of Conway (1947), and uric acid was determined colorimetrically according to the method of Brown (1945). Periodically, blood samples were drawn from the mother by severing the tail, and comparisons of urea and ammonia were made with non-pregnant females and males. The measurements of ammonia and urea production by the adult snake were obtained by confining the snake in a glass container, clearing the cloaca by pressure, and washing the snake with 100 ml. water which served as diluent for the excreta.

Embryonic tissues were obtained by serial removal from the mother after anaesthetization with Nembutal (Clark, 1937). The tissues for analysis were weighed on a Roller-Smith Balance and a quantity of water was added which would bring the urea or uric acid to a concentration suitable for accurate determination by the techniques employed. The tissues were then homogenized in a Potter homogenizer, and aliquots were taken for assay of ammonia, urea and uric acid.

Dry weights were obtained by treatment in a vacuum oven at 30° C. at approximately 50 mm. pressure to constant weight. Protein assays were made according

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to the method of Willits, Coe & Ogg (1949). Ashing was accomplished by preliminary heating in a muffle furnace for 72 hr. at 500° C., and by direct heating in a porcelain crucible over a gas flame (*ca.* 1000° C.) to constant weight.

Twelve female garter snakes provided embryos for the analyses reported below. They were collected locally during May and June. While in captivity they were fed earthworms and were kept at ordinary laboratory temperatures. The procedure for determining embryonic age is described elsewhere (Clark, Florio & Hurowitz, 1955).

The determination of transfer of ammonia from urea to uric acid was accomplished by injection of a known quantity of ^{15}N urea and subsequent recovery of ^{15}N from excreted uric acid. Injections of 1·5 ml. of 100 mg.% ^{15}N urea (15% excess of ^{15}N) were made intraperitoneally in five males and five pregnant females and into the yolk sacs of embryos (0·75 ml. into each of two) of five pregnant females. Excreta were collected at 24 hr. intervals by washing cloacal contents into a vial. The excreta were freed of ammonia and urea by repeated washing and centrifugation; uric acid of the residue was dissolved in 0·25% Li_2CO_3. The dissolved uric acid was degraded by the method of Edson & Krebs (1937), consisting of oxidation by MnO_2 at 40° C., alkaline hydrolysis and acid hydrolysis in a steam bath, enzymic hydrolysis of urea, collection of ammonia in 0·05 HCl, and oxidation of NH_4Cl to N_2 gas *in vacuo* (Rittenberg, 1947). Gas samples were analysed spectrometrically for $^{29}N/^{28}N$ ratio, which was compared with the ratio obtained for gas similarly derived from chemically pure uric acid. (The authors are indebted to Dr S. Friedland and Mr George Strakna for co-operation in making the analyses.)

RESULTS

Growth. Nitrogen metabolism during embryonic development is of necessity a composite of activities related to growth, differentiation and maintenance. To provide a frame of reference for the data on excretion and to evaluate the role of the placenta in nutrition and excretion, some aspects of protoplasmic increase will be considered.

The curve of wet-weight increase of the embryo is presented in Fig. 1*a*. Relationship of wet weight to dry weight is described in Table 1. These data confirm the common observation that early in development, wet weight increases rapidly, and in later stages, more slowly, whereas the reverse is true for dry weight. Rate of increase for embryonic dry weight in terms of k values is stated in Table 2.

Because the garter snake embryo develops from a highly telolecithal egg, but establishes a placental union with the mother, distinction between the ovarian and placental roles in nutrition is difficult. It was pointed out (Clark *et al.* 1955) that water, and probably amino-acids, pass the placenta. Some evidence with regard to protein exchange is provided by comparison of total initial protein with the amount found in neonates (Table 1).

Wet and dry weights of yolk and yolk sac were obtained over the entire incubation period; ratio of water and dry substance remained constant throughout the

Table 1. *Dry weight in relation to wet weight in garter snake development*

Age (days)	Embryo (mg.)				Yolk and yolk sac (mg.)			Total. Embryo and adnexa (mg.)			
	Wet	Dry	Protein	Ash	Wet	Dry	Protein	Wet	Dry	Protein	Ash
0	—	—	—	—	490	255	132	490	255	132	—
0	—	—	—	—	453	221	111	453	221	111	—
3	—	—	—	—	—	—	—	462	228	—	17·1
3	—	—	—	—	—	—	—	680	294	—	24·1
7	—	—	—	—	—	—	—	469	221	131	—
7	—	—	—	—	—	—	—	659	285	117	—
21	—	—	—	—	—	—	—	665	198	79	—
21	—	—	—	—	—	—	—	453	235	117	—
22	158	12	—	—	601	262	107	759	274	—	—
25	219	18	9	—	735	325	150	954	343	—	—
25	220	18	11	—	620	322	—	840	341	—	—
26	249	21	11	—	731	329	144	980	350	—	—
35	307	24	15	—	691	299	136	998	323	151	—
35	242	—	—	—	708	314	88	1006	311	122	—
35	—	—	—	—	687	159	45	1305	350	135	—
38	366	28	15	—	729	338	150	1095	366	165	—
49	562	59	29	—	647	312	123	1209	371	152	—
50	581	50	30	—	633	297	78	1214	347	108	—
50	566	52	—	4·6	614	281	—	1180	333	—	—
51	625	43	31	—	584	316	—	1209	359	—	—
57	758	64	41	—	461	210	—	1219	274	—	—
57	768	72	—	10·2	232	106	—	1000	178	—	—
59	824	96	53	—	531	237	131	1355	333	184	—
59	820	66	—	—	455	208	—	1275	274	—	—
59	816	64	—	7·8	459	249	—	1275	313	—	—
67	1098	129	81	—	213	97	—	1311	226	—	—
67	1004	115	—	16·1	243	111	—	1247	226	—	—
70	1246	189	91	—	328	151	87	1574	340	178	—
70	1244	223	—	24·8	0	0	0	1244	223	—	—
70	1219	247	147	—	0	0	0	1219	247	147	—
70	1287	244	—	26·8	0	0	0	1287	244	—	—
70	1242	240	153	—	0	0	0	1242	240	153	—
70	1303	231	143	—	0	0	0	1303	231	143	—

Table 2. *k values for growth rate and rates of accumulation of excreted nitrogen*

(From the equation $W = Ae^{kt}$, where W = weight, A = ln W when $T = 0$, e = base of natural logarithms, T = time (age). k value with period covered (days) shown in parentheses.)

Wet weight	0·197 (0–25) 0·055 (25–53) 0·010 (55–72)
Dry weight	0·047 (20–57) 0·094 (57–71)
Total N	0·11 (0–33) 0·028 (33–72)
Ammonia N	0·050 (0–72)
Urea N	0·092 (0–32) 0·032 (33–72)
Uric acid N	0·073 (0–72)

gestation period (water = 54·3 ± 3·0%). The dry weight of the yolk and yolk sac rises to a peak on the 25th day, which is maintained until the 50th day, after which the dry substance becomes depleted with resorption of the yolk sac. Intrauterine dependence on stored yolk by garter snake embryos is in contrast with the sparing of yolk for post-partum nutrition of *Vipera berus* (Bellairs, Griffiths & Bellairs, 1955).

Table 3. *Excreta recovered from total garter snake egg in mg. nitrogen*

Age (days)	Embryo weight (mg.)	NH_3	Urea	Uric acid	Total
Unfert.	—		0·047	—	0·047 est.
6	—		0·021	—	0·021 est.
17	—		0·029	—	0·029 est.
18	45		0·045	—	0·045 est.
22	100	0·044	0·161	0·006	0·211
33	360	0·037	0·529	0·016	0·582
34	269	0·035	0·290	0·010	0·335
35	273	0·074	0·452	0·016	0·542
38	401		0·492	—	0·522 est.
41	373	0·093	0·409	0·024	0·526
43	434	0·094	0·374	0·035	0·503
43	459	0·120	0·527	0·031	0·678
45	533	0·069	0·410	0·016	0·495
49	565	0·052	0·517	0·018	0·587
50	636	0·120	0·513	0·039	0·672
50	907	0·133	—	—	—
60	901	0·094	0·643	0·074	0·811
60	984	0·080	0·690	0·087	0·857
61	904	0·238	0·775	0·064	1·077
61	1308	0·276	0·725	0·100	1·101
63	1193	0·089	0·880	0·036	1·005
65	929	0·084	0·825	0·076	0·985
65	1446	0·124	0·957	0·098	1·179
67	686	0·201	0·775	0·027	1·003
69	1500	0·095	0·867	0·110	1·072
69	1510	0·117	1·030	0·117	1·264
70	994	0·111	0·770	0·104	0·985
72	1000	0·139	0·582	0·120	0·841
72	1406	0·390	0·733	0·390	1·513

Protein analyses (Table 1) suggest that the principal dry substance lost during the early decline is protein; since the protein content of the neonate is greater (approximately 30 mg.) than that originally present, it is apparent that this amount was supplied through the placenta, presumably as amino-acids. Later, it will be shown that the excreta per embryo would require the degradation of approximately 16 mg. protein; hence, a minimum of 45 mg. per embryo will have passed the placenta during the incubation period.

There is apparently a slight increase in ash content of the embryo in comparison with the original store available, both in absolute quantity and in percentage composition (Table 1). This would imply that a small portion of the ash of the embryo is derived by transfer across the placenta; the implication is not construed as denial of a more extensive transplacental traffic of inorganic substances in both directions.

Excretion. The excretory data are recorded in Table 3. Graphic representations of these data in Figs. 1*a* and 1*b* express more clearly interrelationships and trends. Total excreted nitrogen (Fig. 1*a*) follows the pattern of growth, though it will be seen (Table 2) that the rate of accumulation of waste products in the early period of development ($k=0{\cdot}11$) is less than the rate of growth of wet weight ($k=0{\cdot}197$) and greater than that of dry weight ($k=0{\cdot}047$). This would point to a relatively

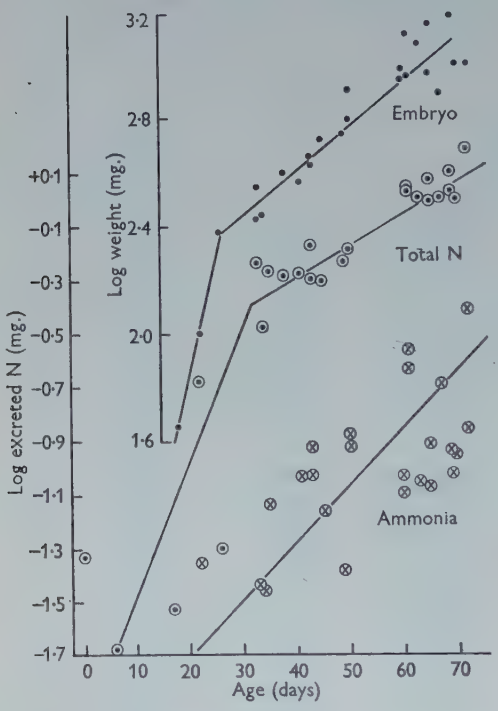

Fig. 1*a*

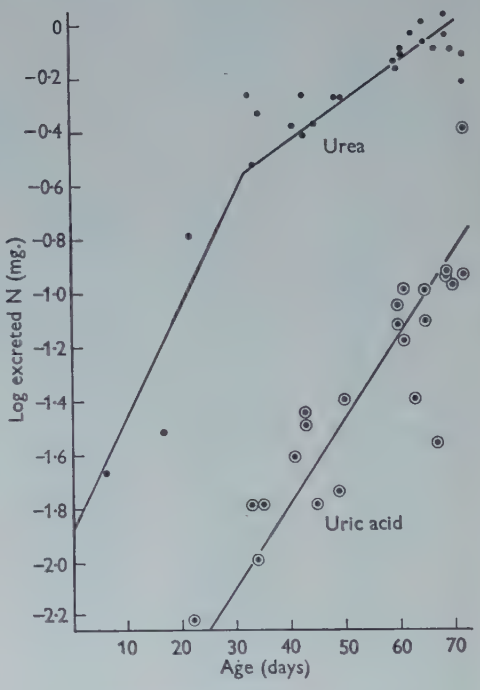

Fig. 2*b*

Fig. 1*a*. Cumulative excreta of the garter snake embryo in relation to growth, semilog plot. Ordinate: upper curve, log wet weight; middle curve, log accumulated excreted N of all types; lower curve, log accumulated ammonia N. Abscissa: age in days. Equations for growth curve: $y=0{\cdot}0945x-0{\cdot}04$ (0–25 days); $y=0{\cdot}022x+1{\cdot}72$ (25–53 days); $y=0{\cdot}0044x+2{\cdot}676$ (55–72 days). Equations for total excreted N are: $y=0{\cdot}048x+8{\cdot}06$ (0–33 days); $y=0{\cdot}012x+9{\cdot}22$ (33–72 days). Equation for ammonia N is: $y=0{\cdot}0215x+7{\cdot}86$ (0–72 days).

Fig. 1*b*. Semilog plot of accumulated urea N and uric acid N. Ordinate and abscissa as in Fig. 1*a*. Equations for urea are: $y=0{\cdot}040x+8{\cdot}24$ (0–32 days); $y=0{\cdot}013x+9{\cdot}06$ (33–72 days). Equation for uric acid N is : $y=0{\cdot}0319x+6{\cdot}91$ (0–72 days).

greater decomposition of protein early in development. When considered on the basis of unit weight, this observation is more apparent. In Fig. 2 it is seen that the rate of excretion per gram of tissue early in development is almost three times the rate at hatching. It would appear, therefore, that protein is being used as an energy source to a greater extent in the early period of development. (Support for this view comes also from study of other reptiles and the chick, in which the relationship described is repeated.) These data alone do not deny the hypothesis

of Needham (1931) that the principal energy sources of development are successively carbohydrate, protein and fat. It would seem, however, that protein should be given serious consideration as a primary energy source in early development.

Ammonia accumulates at a constant rate ($k=0{\cdot}055$); since the rate of accumulation of embryonic wet-weight decreases, it follows that the concentration of ammonia with respect to the embryo increases. Ammonia may be disposed of in

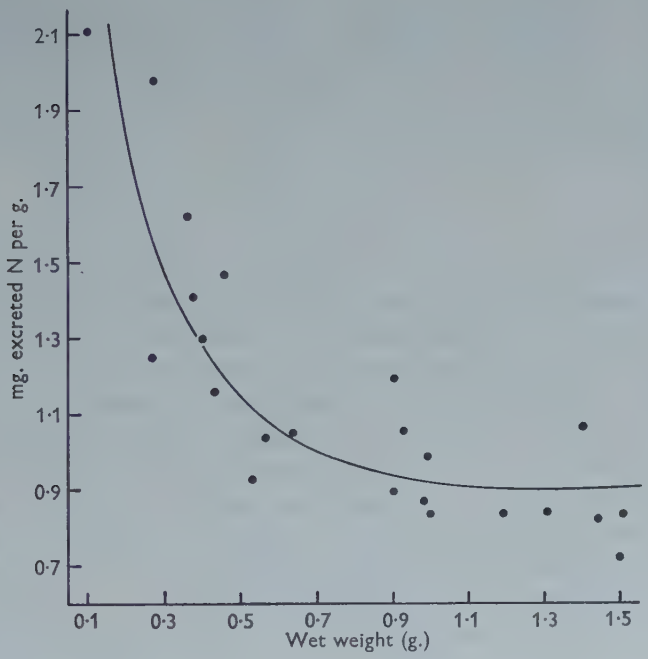

Fig. 2. Rate of excretion of nitrogen in relation to age in the garter snake embryo.

several ways: (1) urea synthesis, (2) uric acid synthesis, (3) transamination, or (4) transplacental migration. Since the concentration in embryonic tissues (and the egg as a whole) increases, it would appear that these methods are inadequate to dispose of the amount of ammonia produced; however, the increase in concentration is obviously not deleterious to the developmental processes.

The rate of uric acid accumulation is also constant; initially very small amounts are detectable, and the rate of accumulation is relatively high ($k=0{\cdot}073$). Uric acid accumulation is apparently contingent upon development of the allantois, since it occurs principally after 3 weeks of development.

Urea accumulates rapidly during the first month ($k=0{\cdot}092$), but after this time at a more moderate rate ($k=0{\cdot}032$). On the basis of nutritional data above, the placenta is presumably functional after about 20 days of development. Since urea is extremely soluble, it is reasonable to suppose that it is lost through the placenta.

Since uric acid accumulation occurs principally after the period of rapid urea accumulation, it is possible that the decline in rate of urea accumulation may, in part, be the result of intra-embryonic conversion to uric acid. A similar interpretation would appear to be appropriate for data obtained by injection of ^{15}N urea (see below).

Table 4. *Comparison of excreta in garter snake and black snake embryos (mg.)*

	Weight (g.) at hatching of birth	Total excreted nitrogen	Total excreted nitrogen per gram	Percentage of total excreted nitrogen		
				Ammonia	Urea	Uric acid
Black snake	7·0	12·55	1·80	20·0	60·0	20·0
Garter snake (recovered)	1·4	1·41	1·00	23·4	60·3	16·3
Garter snake (calculated)	1·4	2·52	1·80	(90·9)		9·1

Regardless of the explanation for the mechanism of decline in urea accumulation, the importance of urea in the excretory pattern of the embryonic garter snake is attested by the similar decline in total nitrogenous waste.

Comparison of the excretory data of the garter snake and the black snake is made in Table 4. The partition of the recovered nitrogenous end products in the two species corresponds quite closely. Excretion per gram is far less in the garter snake (56%). Presuming the same composition of the two neonatal snakes that have attained similar physiological and morphological maturity, it would appear that some of the garter snake excreta are missing. To establish the same excretory rate in the garter snake would require the excretion of 2·52 mg. nitrogen. It would therefore appear that 1·11 mg. per embryo was lost through the placenta; since it is not possible to measure quantitatively the amounts of ammonia and urea which pass the placenta, the two have been combined in Table 4. It will be noted that the relative importance of uric acid in the placental species is reduced. Evidence pertaining to the role of the placenta in embryonic excretion is derived from three sets of data; they are described below.

Transplacental passage of urea

(1) *Blood urea.* A mother carrying fifteen embryos would be expected to receive a minimum of 36 mg. urea during the period of gestation, on the basis of the calculations above. Assuming that all were released during the latter half of the gestation period, the average rate of passage would be of the order of 0·05 mg. urea/hr.—sufficient to cause an increase of approximately 1 mg.% in maternal blood content. Accordingly, urea assays of maternal blood were made throughout gestation, for which males and non-pregnant females served as controls. Average values for males throughout the observation period was 4·24 mg.% (2·95–6·15), and for non-pregnant females, 5·01 mg.% (2·95–6·05); pregnant females during

the first half of the gestation period averaged 5·13 mg.% (3·50–7·40) and during the latter half, 6·93 mg.% (2·50–20·0). These data are suggestive rather than conclusive, but indicate transplacental loss of urea. They support the calculations above, but, because of variability and the demonstrated maternal urease activity (see below), cannot be said to verify them.

(2) *Excreted urea plus ammonia.* It was thought that if urea and ammonia passed through the placenta in the quantities calculated above, and were eliminated without change through the maternal kidneys, they could be recovered in the cloaca. Therefore, towards the end of gestation, two pregnant females and a non-pregnant female, all closely similar in size, were isolated in glass containers. Their combined urea and ammonia output was measured daily over an 8-day period. Average daily output of the two pregnant females was 2·0 mg.; that of the non-pregnant female was 2·2 mg. A third pregnant female was given three injections of 0·8 ml. 500 mg.% urea into the body cavity on alternate days (a total of 12·0 mg. urea). Her average output was 1·9 mg. per day.

Assays of liver and kidney of adults for urease were made, and both showed extensive activity. It was therefore suspected that urea, either artificially supplied or derived from the embryos, is converted to carbon dioxide and ammonia, and the latter may be incorporated into the uric acid molecule.

(3) *Conversion of* ^{15}N *urea.* In order to determine whether urease, shown to be active *in vitro*, was also physiologically active, the procedure outlined above (methods) was carried out. The results of the analysis are shown in Table 5.

Table 5. *Conversion of urea to uric acid (garter snake) after 48 hr.*

	^{15}N injected as urea (converted to ml. gas)	^{15}N recovered from uric acid of excreta (ml. gas)	Atom percentage excess ^{15}N recovered	Percentage ^{15}N injected recovered
Males (intraperitoneal)	0·078	0·045	3·32	57·0
Females (intraperitoneal)	0·078	0·025	1·52	32·0
Females (yolk sac)	0·078	0·017	0·75	22·0

The data point conclusively to a conversion of injected urea to uric acid. Since a quantitative statement would entail collection of 100% excreted uric acid, and preservation of this through the various steps of the process, and measurement of residual ^{15}N in the injected animal, the foregoing data must be regarded as estimates. The estimate of percentage recovery is, however, a conservative one.

It is believed that the injected urea is hydrolysed by urease, and that the resultant ammonia is incorporated into the uric acid molecule. Such a hypothesis explains the data of Table 5, the failure of injected urea ((2) above) to appear as urea, and the relatively slight increase in blood urea of pregnant females. It is not proposed that all of the embryonic urea which escapes into the maternal blood stream is subjected to urease hydrolysis, since a large amount of urea is excreted by the garter snake. The lower percentage conversion of intra-embryonically

injected urea to adult uric acid may point to the operation of the same mechanism within the embryonic confines. Because of the small quantities of uric acid available from an embryo, this problem could not be pursued. However, the fact that a significant percentage of the injected urea was recovered from the maternal excreta demonstrates conclusively the placental role in embryonic excretion.

DISCUSSION

Four aspects of the present data would seem to be of interest: (1) relation of the placenta to embryonic excretion in a reptile, (2) similarity of the mechanism of excretion in this, a viviparous species, to that of the black snake, an ovoviviparous species, (3) the identification of urease in another uric-acid producing animal, and (4) the indication that protein, particularly in early development, may be an important energy source.

The morphological findings of Weekes (1935) with respect to the reptilian placenta invite speculation, but the present data are believed to be the first which demonstrate a placental role in excretion. The concentrations of embryonic urea during the course of gestation are not so high that transplacental passage of urea may be regarded as a physiological or evolutionary requirement. It would rather appear that the extreme solubility of urea makes it inevitable that some must escape from the embryonic confines. It could hardly be argued that the placenta has survival value on account of excretory advantages alone.

No evidence has come to light which would indicate a qualitative difference in the basic mechanisms of excretion between the garter snake, a non-cleidoic, and the black snake, a cleidoic form. Since the possible methods of eliminating nitrogen are so limited, a reduction of the relative importance of uric acid is the only change which might reasonably be expected.

If our estimate of the urea passing through the placenta is approximately correct, the placenta cannot be regarded as constituting a barrier to the elimination of urea. The garter snake, then, is an exception to Needham's (1950) theory that the excretory mechanism of the adult vertebrate is that which is imposed upon it by conditions of embryonic development.

The identification of urease in the adult garter snake and the presence of urease throughout the development of the chick (Clark, Fischer & Florio, 1953) suggests that the synthesis of uric acid may be a means of getting rid of the ammonia which is produced by the breakdown of urea. Urease is found in other animals which produce uric acid, namely, in the chick at 4 weeks post-hatching, in various tissues of the mouse and rabbit, and has been shown to be present as well in the rat, cat, dog, mouse and frog (Fitzgerald, 1948) and in *Helix* (Baldwin & Needham, 1935). Since it has been found abundantly in the liver and kidney of the newt and frog (in which its activity would create no serious problem because ammonium salts might readily be eliminated to an aqueous environment or enter into other metabolic pathways), the possibility exists that it arose in the amphibia and has been transmitted genetically to eptilian, avian and mammalian descendants. The

uric acid mechanism in terrestrial animals would then be construed as an evolutionary essential, whose fundamental inception was occasioned by necessity of getting rid of ammonia rather than purines. A functional relationship between urease activity and uric acid synthesis seems sufficiently well established to warrant further pursuit of the problem.

The fact is established that the rate of excretion early in garter snake development is three times greater per unit mass of tissue than that at the end of development; since similar data are available for other vertebrates (unpublished), it would appear that a painstaking analysis of energy sources in both the chick and reptiles might be fruitful.

SUMMARY

1. The garter snake embryo excretes an estimated 2·52 mg. nitrogen, of which 1·4 mg. is recoverable from the embryonic confines. The recovered excreta consist of 16·3% uric acid, 23·4% ammonia and 60·3% urea.

2. The placenta is believed, therefore, to transmit to the mother 1·11 mg. nitrogen per embryo, and it is estimated that it transmits to the embryo approximately 45 mg. of protein as amino-acids.

3. Evidence is presented which suggests that protein may be a principal source of energy, particularly early in development.

4. Growth in terms of wet weight, dry weight, and protein is described.

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STUDIES ON THE TRANSFER OF FERTILIZED MOUSE EGGS TO UTERINE FOSTER-MOTHERS

I. FACTORS AFFECTING THE IMPLANTATION AND SURVIVAL OF NATIVE AND TRANSFERRED EGGS

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INTRODUCTION

In 1890 Walter Heape performed a classical experiment which he described as follows: 'On the 27th April, 1890, two ova were obtained from an Angora doe rabbit which had been fertilised by an Angora buck thirty-two hours previously.... These ova were immediately transferred into the upper end of the fallopian tube of a Belgian hare doe rabbit which had been fertilised three hours before by a buck of the same breed as herself....

'In due course this Belgian hare doe gave birth to six young—four of these resembled herself and her mate, while two of them were undoubted Angoras.'

Heape thus demonstrated that a pregnancy could be artificially induced differing in two respects from a normal pregnancy. First, the Angora young had no blood relationship to their uterine foster-mother or to their foster-siblings. Secondly, their post-conception age differed by over a day from their foster-mother's post-coital phase, and hence from the post-conception age of their foster-siblings.

'The experiment...', wrote Heape, 'was undertaken to determine in the first place what effect, if any, a uterine foster-mother would have upon her foster-children, and whether or not the presence and development of foreign ova in the uterus of a mother would affect the offspring of that mother born at the same time.'

To this may be added another main category of inquiry opened up by Heape's achievement: the effect of the presence and development of foreign ova upon the uterine foster-mother herself. As a single example: was the duration of pregnancy in Heape's recipient rabbit influenced by her precocious fosterlings? For it is certain that either the fosterlings were born prematurely or their foster-siblings were born belatedly.

These three broad categories do not exhaust the range of problems in genetic's embryology, reproductive physiology, immunology and cancer research which can be attacked by the technique of egg transfer. A further reason for interest in the subject is the possibility of practical application to livestock farming. It has been shown that a sexually immature female can be induced by hormone treatment to ovulate an abnormally large number of eggs. These can be fertilized *in situ* and then transferred to a battery of sexually mature recipients. It therefore becomes possible in theory (1) to shorten the generation interval and thus to accelerate the improvement of livestock by selective breeding (see Adams, 1954), and (2) to multiply the

genetic contribution to the breed made by outstanding females, just as artificial insemination can be used to propagate the good qualities of outstanding males. Improvement of methods of storing mammalian eggs also holds out the possibility of transporting an entire herd or flock about the world within the confines of a vacuum flask.

In all the uses to which egg transfer has been put in a variety of mammalian species, the yield of living young obtained has been low. For its full exploitation as a research tool the technique will require to be brought to a high level of reliability in a cheap, fecund and rapidly maturing laboratory animal. As a step towards this end we have made a study of some of the factors affecting the implantation and survival of fertilized mouse eggs after transfer to uterine foster mothers.

The first successful transfer of mouse eggs to a uterine foster mother was performed in 1935 by Little (cited by Bittner & Little, 1937). Subsequent published work includes studies by Fekete & Little (1942), Fekete (1947), Beatty (1951), Runner (1951), Runner & Palm (1953) and Boot & Mühlbock (1953).

MATERIALS AND METHODS

Donors and recipients

As *donors* we used albino females supplied by 'The Mousery', Rayleigh, Essex. They were mated with albino males from the same source. As *recipients* we used miscellaneous adult females homozygous for full colour, mated with male F_1 hybrids between the C3H and C57BL inbred strains. Embryos of donor origin were thus homozygous albinos and could be distinguished at autopsy from native embryos by their eye colour.

For egg transfers from immature donors, ovulation was induced according to the general procedure of Runner & Palm (1953). Priming doses of pregnant mare's serum were usually given on the twenty-seventh and twenty-eighth days of life, and chorionic gonadotrophin was injected at midday on the thirtieth day of life. According to Runner & Palm ovulation is expected to occur 13 ± 2 hr. later, i.e. at about 1 a.m. This coincides with the time of night at which mice usually mate (Snell, Fekete, Hummel & Law, 1940).

The occurrence of mating in both donors and recipients was detected by the presence of a vaginal plug on the following morning. Operations were performed either 2 or 3 days later during the period 10 a.m.–4 p.m. Hence to an accuracy of a few hours either $2\frac{1}{2}$ or $3\frac{1}{2}$ days had elapsed between the mating of female mice and their use as donors and recipients.

Recovery of fertilized eggs

The donors were killed and the gross appearance of the ovaries noted, although exact counts of corpora lutea were not made. Each uterine horn with attached oviduct was placed in a watch-glass in a few ml. of Ringer-phosphate saline (Pannett & Compton, 1924) at room temperature. The eggs were then recovered under a dissecting microscope.

In the case of $2\frac{1}{2}$-day donors, each oviduct was chopped with a mounted surgical needle into short lengths, and each length was stroked with the needle, so that all contained eggs emerged, together with epithelial debris.

In the case of $3\frac{1}{2}$-day donors the eggs were usually already in the uterus, but sometimes in the very last section of the oviduct where it enters the uterus. This terminal section was severed from the rest of the oviduct and emptied by stroking with the needle. The uterine horn was then cut through close to its junction with the oviduct, and the uterine fragment which remained attached to the oviduct was searched for eggs. Finally, a stream of saline was blown from a fine Pasteur pipette through the uterine horn from the vaginal towards the ovarian end. The entire field was then searched for eggs.

As the eggs were found they were immediately transferred in a very fine Pasteur pipette to a solid watch-glass containing a few drops of saline and covered with a glass cover-slip. When the search for eggs was complete, the eggs were marshalled together by gently directing streams of saline towards them. Finally, they were picked up together with a little fluid, using the capillary action of a Pasteur pipette having a very fine terminal section 2–3 cm. in length. The eggs were then ready for injection into the uterus of the recipient.

Transfer of eggs to the host uterus

The recipient was anaesthetized with ether and a dorsal transverse skin incision was made to the left of the mid-line. On retraction of the margins of the incision the left ovary could usually be seen through the semi-transparent abdominal wall, which was then incised at the point where it overlay the ovary. The ovarian fat pad was seized with forceps and pulled through the incision, carrying with it the ovary, the oviduct, and the upper part of the left uterine horn. The appearance of the ovary was recorded and the uterine horn was then punctured near the top with a hypodermic syringe fitted with a needle of 0·47 mm. external diameter. Through this puncture the end of the pipette containing eggs was introduced, with the tip pointing away from the oviduct. The saline and contained eggs were injected into the uterus by gently squeezing the bulb of the pipette.

After the ovary, oviduct and uterine horn had been pushed back with a probe into the abdominal cavity the skin incision was closed with cotton sutures. The incision in the abdominal wall was left unsutured, and was invariably found at autopsy to have healed, sometimes, however, with adhesion to the left ovarian fat pad.

THE PLAN OF THE EXPERIMENT

We have been concerned mainly to investigate three factors affecting the survival of transferred eggs: the relative stages post-coitum of donor and recipient, the trauma suffered by the recipient from the surgical procedure, and the sexual maturity or immaturity of the donor. The number of eggs injected was varied, so that we have been able to study this factor also.

Donors and recipients were used either $2\frac{1}{2}$ or $3\frac{1}{2}$ days post-coitum. All four combinations of donor and recipient stages were tested, using induced ovulation of

immature donors. The most successful combination ($3\frac{1}{2}$-day donor, $2\frac{1}{2}$-day recipient) was repeated, using natural ovulation from mature donors. This combination was also repeated, together with the least successful ($2\frac{1}{2}$-day donor, $3\frac{1}{2}$-day recipient), using recipients in which pseudo-pregnancy had been induced by mating to sterile vasectomized males. In addition, 'dummy' transfers (saline without eggs) were done on both $2\frac{1}{2}$- and $3\frac{1}{2}$-day recipients. Finally, intended recipients for which no donors were available were killed and examined after the same lapse of time as operated mice, thus providing a series of unoperated controls. The general plan of the experiment is set out in Table 1.

Table 1. *Plan of experiment*

Group	Post-coital stage (in days) of		State of donor	State of recipient
	Donor	Recipient		
Unoperated controls	—	—	—	—
o→$3\frac{1}{2}$	—	$3\frac{1}{2}$	—	Pregnant
o→$2\frac{1}{2}$	—	$2\frac{1}{2}$	—	Pregnant
$2\frac{1}{2}$→$3\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$	Immature, induced ovulation	Pregnant
$2\frac{1}{2}$→$2\frac{1}{2}$	$2\frac{1}{2}$	$2\frac{1}{2}$	Immature, induced ovulation	Pregnant
$3\frac{1}{2}$→$3\frac{1}{2}$	$3\frac{1}{2}$	$3\frac{1}{2}$	Immature, induced ovulation	Pregnant
$3\frac{1}{2}$→$2\frac{1}{2}$	$3\frac{1}{2}$	$2\frac{1}{2}$	Immature, induced ovulation	Pregnant
Mature, $3\frac{1}{2}$→$2\frac{1}{2}$	$3\frac{1}{2}$	$2\frac{1}{2}$	Mature, natural ovulation	Pregnant
$2\frac{1}{2}$→$3\frac{1}{2}$ (vas.)	$2\frac{1}{2}$	$3\frac{1}{2}$	Immature, induced ovulation	Pseudo-pregnant
$3\frac{1}{2}$→$2\frac{1}{2}$ (vas.)	$3\frac{1}{2}$	$2\frac{1}{2}$	Immature, induced ovulation	Pseudo-pregnant

Transfers were done into one uterine horn only (the left), so that its contents at autopsy could be assessed against those of the unoperated, or control, horn. 14 days after operation ($16\frac{1}{2}$ or $17\frac{1}{2}$ days post-coitum) all recipients were killed and each implantation in both horns was classified as 'live alien embryo', 'live native embryo' or 'dead resorbing embryo'. On a few occasions several of the recipients were found to contain only dead resorbing embryos. Such pregnancies, numbering nine in all, were reckoned to be abnormal, and have been omitted from the data presented in this paper.

Embryos which die soon after implantation (i.e. after the fifth and before the tenth day) may be completely resorbed before autopsy and hence escape notice. Where necessary to avoid confusion we shall use the phrase 'successful implantations' to denote those which survive this period. Our later references to post-implantational mortality should be taken to mean 'post-implantational mortality detectable by our methods and criteria'.

DESCRIPTION AND ANALYSIS OF RESULTS

Control data

As shown in Table 2, thirty-one out of fifty-four unoperated control females became pregnant. They were found to have 210 implantations distributed between the two horns, an average of 6·8 per female. Thirty-three of these were resorbing, making an overall resorption rate of 15·7%.

Table 2. *Control data on unoperated mice*

No. of mice showing vaginal plugs	No. of pregnancies		No. of embryos in						Grand total
			Right horn			Left horn			
			Live	Resorbing	Total	Live	Resorbing	Total	
54	31	Total	95	14	109	82	19	101	210
		Av. per pregnancy	3·1	0·5	3·5	2·6	0·6	3·3	6·8
		% resorbing		12·8			18·8		

Table 3. *Uninjected horns of operated mice compared with control data on unoperated mice*

	No. of mice showing vaginal plugs	No. of pregnancies		No. of implantations			% resorbing
				Alive	Resorbing	Total	
Unoperated mice	54	31	Total	177	33	210	15·7
			Av. per horn*	2·9	0·5	3·4	
Operated mice	202	129	Total	411	65	476	13·7
			Av. per horn†	3·2	0·5	3·7	

* Both horns combined. † Uninjected horns only.

Considering the two sides separately, the right horns had an average of 3·5 implantations of which 12·8% were resorptions, while the left horns had an average of 3·3 implantations of which 18·8% were resorptions. Neither of these differences approaches statistical significance. There was no correlation between the two sides in the number of implantations ($r = -0{\cdot}025$, $n = 31$). But there was a large and statistically highly significant positive correlation between the two sides in the number of resorptions ($r = +0{\cdot}651$, $n = 31$).

Systemic effects of surgical interference

In Table 3 we give comparative data on operated and unoperated mice, from which we can inquire whether or not the operation has generalized effects upon the recipient's reproductive processes apart from the local effects upon the injected uterine horn.

(1) *Does the operation affect the pregnancy rate?*

The proportion of all operated mice which became pregnant (excluding those mated with vasectomized males) was 129/202 = 64% as compared with 31/54 = 57% in the unoperated controls. The difference is not significant. We may therefore exclude all non-pregnant mice from further consideration, since the pregnancy rate is evidently controlled by factors extraneous to the technique of egg transfer itself.

(2) *Does the operation affect implantation in the uninjected uterine horn?*

The mean number of implantations in the right (uninjected) horns of all operated mice (excluding those mated with vasectomized males) was $3{\cdot}69 \pm 0{\cdot}16$ as compared with $3{\cdot}39 \pm 0{\cdot}21$ for the unoperated controls (average of both horns). The former figure has received a trivial increment from the transmigration* of four embryos (see Table 7) from the left into the right horn, and may possibly also include one or two resorptions derived from the same source. We can, however, conclude that the operation does not substantially reduce the implantation rate in the uninjected horn. We shall later come to an important, although quantitatively small, qualification of this conclusion, to the effect that when large numbers of eggs are transferred some inhibition of implantation in the uninjected horn appears to occur.

(3) *Does the operation affect post-implantational mortality in the uninjected horn?*

Of 476 implantations in the uninjected horns of the operated mice, sixty-five, or 13·7%, were resorbing, as compared with 15·7% in the unoperated controls (both horns combined).

Thus any effect on post-implantational mortality which may result from the operation must plainly be confined to the injected horn.

Local effects of surgical interference

(1) *Evidence from dummy transfers*

It was in order to assess the effects on the injected horn of the surgical trauma involved in egg transfer that we did the dummy transfers of saline without eggs. The relevant data are summarized in Table 4.

The left-hand side of the table shows that the injection of saline alone resulted in the loss before, during or shortly after implantation of about one-third of the recipient's own eggs, whether the operation was performed $2\frac{1}{2}$ or $3\frac{1}{2}$ days post-coitum. Since at the $2\frac{1}{2}$-day stage the recipient's eggs are still in her oviduct, the effect presumably acts, not directly upon the eggs, but upon the uterine horn, reducing the facility with which it subsequently forms or maintains implantation sites.

* In a publication on the transmigration of fertilized mouse eggs (McLaren & Michie, 1954) based on an earlier stage of the present work, we overlooked a previous finding by Runner (1951) of one transmigrant among eighty embryos examined. Runner's embryos were derived from eggs recovered from the donor immediately after ovulation and injected into the right ovarian capsule of the recipient.

Table 4. *The effects of dummy transfers*

Recipients		Average no. of successful implantations			Resorptions as percentage of successful implantations	
Stage	Pregnancies	Right horn (uninjected)	Left horn (injected)	Left horn as percentage of right	Right horn (uninjected)	Left horn (injected)
2½ days	21	3·6	2·5	68	17·1	15·4
3½ days	22	3·8	2·5	64	19·0	33·3

Such uterine damage could act in one of two possible ways. It could reduce the effective number of available implantation sites, thus lowering the upper limit to the number of implantations in the horn. This would decrease the frequency of large numbers of implantations, increase the frequency of intermediate numbers, and leave the frequency of small numbers unaffected, resulting in a skew frequency distribution. Or it could reduce impartially each egg's *chance* of successfully implanting, reducing the mean of the frequency distribution without materially affecting its shape. The observed distribution, shown in Table 5, indicates that the

Table 5. *Frequency distributions of the number of successful implantations in the right (uninjected) and left (injected) horns of recipients of dummy transfers. Results from 2½- and 3½-day recipients were in good agreement and have been combined.*

No. of implantations	0	1	2	3	4	5	6	7	Total
Frequency: Right horns	—	5	7	8	8	7	6	2	43
Left horns	5	10	7	8	7	6	—	—	43

second view is to be preferred. Further support for this view is lent by results from egg transfers to be described later; when large numbers of eggs were injected, the deficit caused by the surgical interference was more than made up, so that the average number of implantations in the left horn was actually greater than normal.

The right-hand side of Table 4 shows that the injection of saline had no effect on the resorption rate in 2½-day recipients, but that it substantially increased the rate in 3½-day recipients.

(2) *Evidence from egg transfers*

We kept fairly detailed notes of all operations, and have consequently been able to make a rough classification of them as either 'traumatic' or 'non-traumatic'. 'Traumatic' operations included those in which air was injected into the uterine horn, or the uterine horn was punctured several times or seized with forceps. The various series of transfers differed in the incidence of 'traumatic' operations. This is because it was not possible to arrange for them to be exactly contemporaneous, although they overlapped in time. The work extended over a period of more than a year, during which time our surgical technique was continually improving.

Table 6 shows the recorded incidence of 'traumatic' operations, and the range of time occupied by each series.

In Table 7 we have set out the full results of each series, tabulating 'traumatic' and 'non-traumatic' operations separately. The results of the various series of egg transfers confirm and extend the conclusions derived from the dummy transfers.

Table 6. *Temporal distribution of the different types of transfer and of the incidence of 'traumatic' operations*

Type of transfer	1954 Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	1955 Jan.	Feb.	Mar.	Apr.	May	June
$0 \rightarrow 3\frac{1}{2}$						—	—	—							
$2\frac{1}{2} \rightarrow 3\frac{1}{2}$				—	—	—	—								
$3\frac{1}{2} \rightarrow 3\frac{1}{2}$	—	—	—												
$0 \rightarrow 2\frac{1}{2}$						—	—	—	—	—	—	—	—	—	—
$2\frac{1}{2} \rightarrow 2\frac{1}{2}$	—	—	—	—	—	—	—	—							
$3\frac{1}{2} \rightarrow 2\frac{1}{2}$					—	—	—								
$3\frac{1}{2} \rightarrow 2\frac{1}{2}$ (mature donors)							—	—	—	—	—	—	—	—	—
'Traumatic' / total	$\frac{2}{7}$	$\frac{1}{7}$	$\frac{3}{10}$	$\frac{3}{6}$	$\frac{5}{14}$	$\frac{6}{17}$	$\frac{1}{16}$	$\frac{4}{24}$	$\frac{1}{7}$	$\frac{1}{6}$	$\frac{0}{3}$	$\frac{0}{2}$	$\frac{0}{1}$	$\frac{1}{7}$	$\frac{0}{2}$

Table 7

Group	Type of operation	No. of pregnancies	Av. no. of eggs injected	Total no. of embryos in: Right horn (uninjected) Native	Alien	Re-sorbing	Total	Left horn (injected) Native	Alien	Re-sorbing	Total
$0 \rightarrow 3\frac{1}{2}$	Traumatic	2	—	9	—	2	11	3	—	1	4
	Non-traumatic	20	—	59	—	14	73	33	—	17	50
$2\frac{1}{2} \rightarrow 3\frac{1}{2}$	Traumatic	6	16·7	18	0	2	20	7	2	9	18
	Non-traumatic	8	15·4	21	0	3	24	25	0	6	31
$3\frac{1}{2} \rightarrow 3\frac{1}{2}$	Traumatic	4	4·5	10	0	0	10	3	0	10	13
	Non-traumatic	15	7·2	57	1	1	59	24	10	11	45
$0 \rightarrow 2\frac{1}{2}$	Traumatic	3	—	12	—	3	15	1	—	0	1
	Non-traumatic	18	—	51	—	10	61	43	—	8	51
$2\frac{1}{2} \rightarrow 2\frac{1}{2}$	Traumatic	7	10·6	27	0	1	28	10	1	6	17
	Non-traumatic	9	9·9	26	1	6	33	20	9	6	35
$3\frac{1}{2} \rightarrow 2\frac{1}{2}$	Traumatic	5	13·4	18	0	0	18	4	8	14	26
	Non-traumatic	15	9·2	42	2	10	54	25	28	12	65
Mature, $3\frac{1}{2} \rightarrow 2\frac{1}{2}$	Traumatic	1	10·0	5	0	2	7	3	1	0	4
	Non-traumatic	16	9·2	52	0	11	63	23	34	15	72

Thus, even 'non-traumatic' transfers to $3\frac{1}{2}$-day recipients caused a substantially increased resorption rate in the injected horn. The increase was still greater in 'traumatic' operations. The relative indifference of the $2\frac{1}{2}$-day uterus to surgical trauma is also confirmed; 'non-traumatic' transfers to $2\frac{1}{2}$-day recipients did not result in increased resorption rates in the injected horn. The infliction of more

serious injury on the $2\frac{1}{2}$-day uterus can, however, cause post-implantational mortality, as shown by the general tendency to increased resorption rates in 'traumatic' transfers to $2\frac{1}{2}$-day recipients.

The reduction in the average number of successful implantations, which was shown to result from the transfer of saline both to $2\frac{1}{2}$- and to $3\frac{1}{2}$-day recipients, is confirmed by the various series of egg transfers. In these series we must assess this reduction from the number of *native* embryos in the two horns, since the total number of implantations has received an increment from the transferred eggs. A deficit of native embryos in the injected horn is a general feature of the egg-transfer series, and it is evident that the deficit is greater in 'traumatic' operations; the yield of alien embryos is also reduced in these operations.

In addition it can be seen that, for each recipient stage, the reduction of the number of native embryos in the injected horn is greater in transfers of $3\frac{1}{2}$- than of $2\frac{1}{2}$-day eggs, i.e. in those series which give the higher yields of alien embryos. This suggests that there is competition between transferred and native eggs, a suggestion which is fully confirmed by facts which will be presented later. The effect is still apparent when attention is confined to the series which give no increased resorption rates in the injected horn ('non-traumatic' transfers to $2\frac{1}{2}$-day recipients), so most or all of the competition occurs before, during or shortly after implantation.

In order to make the various series more directly comparable, 'traumatic' operations will be excluded from further consideration, and subsequent analysis will be based solely on the results of 'non-traumatic' operations.

The effect of the relative post-coital stages of donor and recipient upon the yield of live embryos from transferred eggs

In Table 8 we have abstracted from Table 7 the yields of alien embryos from 'non-traumatic' transfers in the different series. It can be seen that the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ combination gave about twice the yield obtained from the $3\frac{1}{2} \rightarrow 3\frac{1}{2}$ and $2\frac{1}{2} \rightarrow 2\frac{1}{2}$

Table 8. *The yields of alien embryos obtained with different post-coital stages of donor and recipient ('traumatic' operations excluded)*

State and stage of donor	Stage of recipient	Av. no. of eggs injected	No. of pregnancies	No. of alien embryos	Average no. of alien embryos per pregnancy
Immature $2\frac{1}{2}$-day	$3\frac{1}{2}$-day	15·4	8	0	0·0
Immature $3\frac{1}{2}$-day	$3\frac{1}{2}$-day	7·2	15	11	0·7
Immature $2\frac{1}{2}$-day	$2\frac{1}{2}$-day	9·9	9	10	1·1
Immature $3\frac{1}{2}$-day	$2\frac{1}{2}$-day	9·2	15	30	2·0
Mature $3\frac{1}{2}$-day	$2\frac{1}{2}$-day	9·2	16	34	2·1

combinations; the $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ combination yielded no live embryos from the transfers summarized in the table in spite of the much larger average number of eggs injected. It is, however, possible to obtain live embryos from this combination, since two such were found in a single recipient of a 'traumatic' transfer (see Table 7).

Part of the explanation of these differences in yield can be sought in the occurrence, mentioned above, of competition between native and transferred eggs. On this view, where transferred and native eggs differ in developmental stage, the implantation of the precocious group hinders the subsequent implantation of their backward foster-siblings. Competition of this type would bear most heavily upon $2\frac{1}{2}$-day eggs transferred to $3\frac{1}{2}$-day recipients.

The occurrence of competition of this type can be independently demonstrated, and the stage at which it operates established, as follows:

In the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ series we calculate the coefficient of correlation between alien and native embryos in the injected horn. We find $r = -0{\cdot}375$, $n = 31$, $P < 0{\cdot}05$, which indicates the occurrence of competition. To determine the stage at which it mainly occurs, we exclude all post-implantational death by recalculating the correlation solely from those females with no resorptions in the injected horn. We find $r = -0{\cdot}468$, $n = 19$, $P < 0{\cdot}05$. We therefore conclude that the greater the number of precocious alien implantations, the fewer native eggs are able successfully to implant in the same horn. The possibility that some post-implantational competition occurs is, of course, not excluded. Fawcett, Wislocki & Waldo (1947) transplanted fertilized mouse eggs to the anterior chamber of the eye and concluded from their results that 'ova are capable of developing in close proximity only until the most precocious among them begins to implant when it sets up a sphere of influence which renders the immediate vicinity inimical to the further development of other eggs'.

The two synchronous combinations show no effect of this kind, nor does the material of Runner (1951), who performed synchronous egg transfers to the ovarian capsule immediately after ovulation. It is possible that competition is only important under conditions of crowding. Alternatively, the implantation of mouse eggs may render the uterus refractory to the *subsequent*, but not to the simultaneous, implantation of other eggs.

Is competition sufficient to explain the whole of the observed differences in yield?

In order to discover whether the relative stages of transferred eggs and *recipient uterus*, in addition to the relative stages of transferred and native eggs, was playing a part, we made further tests of the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ and $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ combinations, using recipients which had been mated with vasectomized males. The results were thus uncomplicated by the presence of native embryos, all eggs being of donor origin. They were also uncomplicated by undue surgical trauma, for it so happened that all operations of this type were 'non-traumatic'.

The results, set out in Table 9, show a large superiority of the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ over the $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ combination. The superiority is not connected with the greater average number of eggs injected in the former series, since the eight implantations were derived from three successful transfers averaging only eight eggs per transfer. Hence part of the difference in yield between $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ and $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ transfers is independent of the presence of competing native eggs.

Table 9. *Comparison of two combinations of donor and recipient post-coital stages, using recipients mated with vasectomized males*

Av. no. of eggs	Donor	Recipient	No. of recipients	Right horn		Left horn		Total implantations	Live embryos per recipient
				Live embryos	Resorptions	Live embryos	Resorptions		
8·9	$2\frac{1}{2}$-day	$3\frac{1}{2}$-day	14	0	0	1	0	1	0·07
10·0	$3\frac{1}{2}$-day	$2\frac{1}{2}$-day	7	1	1	5	1	8	0·86

Comparison of artificially ovulated eggs from immature donors with naturally ovulated eggs from mature donors

Table 10 gives comparative data on the results from mature and immature donors (excluding 'traumatic' operations). It is clear that the viability of artificially ovulated eggs from immature donors was not inferior to that of naturally ovulated eggs from mature donors. The two $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ series gave results which were similar in all respects, and in subsequent sections they will be combined.

Table 10. *Comparison of transfers from immature and mature donors ($3\frac{1}{2}$ days $\rightarrow 2\frac{1}{2}$ days)*

State of donor	No. of pregnancies	Av. no. of eggs injected		Number of embryos in							
				Right horn (uninjected)				Left horn (injected)			
				Native	Alien	Resorbing	Total	Native	Alien	Resorbing	Total
Immature (induced ovulation)	15	9·2	Total	42	2	10	54	25	28	12	65
			Av. per pregnancy	2·8	0·13	0·67	3·6	1·7	1·9	0·80	4·3
Mature (natural ovulation)	16	9·2	Total	52	0	11	63	23	34	15	72
			Av. per pregnancy	3·3	0·00	0·69	3·9	1·4	2·1	0·93	4·5

The frequency distribution of alien embryos

Fig. 1 shows the thirty-one pregnancies of the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ series arranged in a frequency diagram according to the yields of alien embryos obtained from them. By far the commonest class is that of zero yield, suggesting that over and above the random loss of individual eggs there is a second and distinct phenomenon of whole inoculum loss. In order to make the frequency of zero yield fit a smoothed curve drawn through the rest of the distribution on the assumption of random loss, we should have to discard about ten of the observations in the zero class, assigning them to the category of whole inoculum loss. We conclude therefore that something in the region of one-third of all transfers fail through loss of the whole inoculum of eggs as a unit.

The effect of the number of eggs injected

In Table 11 the results summarized in Table 10 are tabulated according to the number of eggs injected. The same information, together with data from the $0 \rightarrow 2\frac{1}{2}$ series, is presented graphically after appropriate grouping in Fig. 2. The following points stand out:

(1) The average number of successful implantations of all sorts in the injected horn is less than that for unoperated controls by about 1, when *no* eggs are injected (dummy transfers). From this level it rises linearly by increments of about 0·2 for each additional egg injected, finally attaining a value of about 2 more than that found in unoperated controls. Thus, over the range which we tested, there is no evidence of a 'ceiling' to the number of eggs which can implant in one uterine horn.

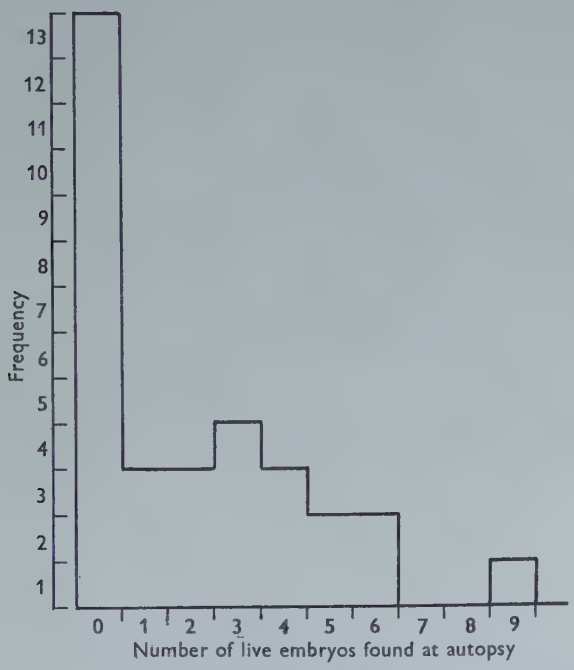

Fig. 1. Frequency distribution of the number of alien embryos in the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ series.

(2) But the average number of successful implantations in the two horns together does appear to reach a 'ceiling'. When large numbers of eggs are injected, the number of implantations in the *uninjected* horn decreases, so that the average number of implantations for the two horns together does not rise above 8·5. This effect will be further discussed in the next section.

(3) The average number of alien embryos in the injected horn, starting from zero in the dummy transfers, rises linearly by increments of 0·225 for each additional egg injected. This is what we should expect if each alien egg had a $22\frac{1}{2}\%$

Table 11. *Data on 'non-traumatic' $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ operations, arranged according to number of eggs injected*

No. of eggs injected	Donor (I = immature M = mature)	No. of embryos in							
		Right horn (uninjected)				Left horn (injected)			
		Native	Alien	Re-sorbing	Total	Native	Alien	Re-sorbing	Total
5	I	4	0	2	6	3	0	0	3
5	I	4	0	2	6	0	0	2	2
5	I	2	0	1	3	1	3	1	5
5	M	1	0	1	2	5	0	0	5
6	I	1	0	1	2	3	1	0	4
6	I	4	0	0	4	1	4	0	5
6	M	2	0	0	2	1	1	1	3
7	I	4	0	1	5	0	0	0	0
7	M	2	0	1	3	0	0	0	0
7	M	5	0	0	5	3	0	0	3
8	I	4	0	0	4	4	1	0	5
8	I	2	1	0	3	2	4	1	7
8	M	6	0	0	6	4	0	0	4
8	M	7	0	2	9	0	4	0	4
8	M	3	0	1	4	2	4	0	6
9	I	3	0	2	5	1	3	0	4
9	M	3	0	3	6	1	0	3	4
9	M	6	0	1	7	0	0	0	0
10	I	3	1	0	4	0	5	0	5
10	I	2	0	0	2	4	0	0	4
10	M	2	0	0	2	1	6	0	7
10	M	3	0	0	3	0	2	3	5
10	M	2	0	0	2	3	2	3	8
11	I	3	0	0	3	2	0	0	2
11	M	3	0	1	4	1	0	2	3
11	M	3	0	0	3	1	3	0	4
12	M	2	0	1	3	1	3	0	4
14	I	2	0	0	2	1	2	1	4
16	I	3	0	1	4	2	0	2	4
16	M	2	0	0	2	0	9	3	12
18	I	1	0	0	1	1	5	5	11

chance of survival irrespective of the number injected. Over the range which we tested there is no tendency for the yield per injected egg to fall off when large numbers are injected, i.e. no evidence of a 'ceiling' to the yield of alien embryos that can be obtained from one horn. In Table 12 a regression analysis of these data is shown.

(4) The average number of native embryos in the injected horn shows a concomitant decline at an overall rate of about 0·09 per additional egg injected. The corresponding regression analysis, set out in Table 13, shows that the effect is significant, thus confirming that the transferred eggs hinder the successful implantation or subsequent survival of the native eggs in the same horn.

(5) The total number of live embryos (natives + aliens) in the injected horn shows a steep initial rise from the level found in recipients of dummy transfers to a level somewhat higher than that found in unoperated mice, but thereafter rises slowly.

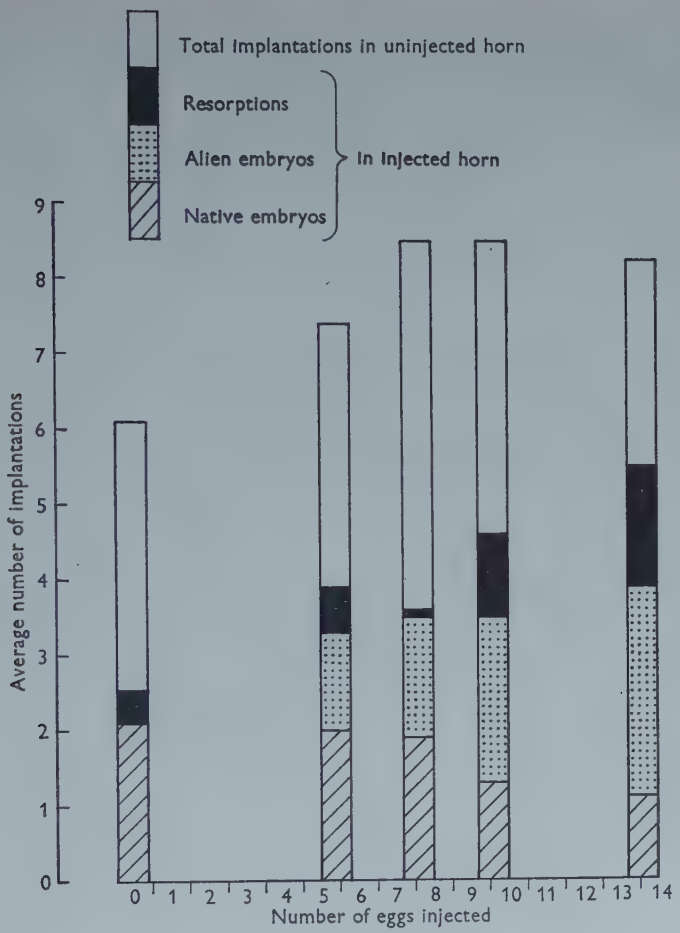

Fig. 2. The results of injecting different numbers of eggs in $3\frac{1}{2}$-day→$2\frac{1}{2}$-day transfers.

Table 12. *Regression analysis of the relation in the* $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ *series between the number of eggs injected and the number of live alien embryos found at autopsy*

	Sums of squares	Degrees of freedom	Mean square	Mean square ratio
Regression	29·3250	1	29·325	6·1 $P<0·05$
Residual	140·5460	29	4·846	
Total	169·8710	30		

(6) When the average number of implantations in the injected horn begins markedly to exceed the normal level, the resorption rate increases.

The last two points suggest that, although we have found no limit to the number of eggs which can implant in a single uterine horn, we are beginning to approach a limit to the number of implantations which a single horn can keep alive.

Table 13. *Regression analysis of the relation in the* $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ *series between the number of eggs injected and the number of live native embryos found in the injected horn (dummy transfers to* $2\frac{1}{2}$*-day recipients included)*

	Sums of squares	Degrees of freedom	Mean square	Mean square ratio
Regression	10·9509	1	10·951	4·9 $P<0·05$
Residual	107·0491	48	2·230	
Total	118·0000	49		

Possible interaction between uterine horns when the number of implantations in one horn is artificially raised by egg transfer

We have seen that in unoperated controls there was no correlation between the number of implantations in the two uterine horns. This is in accord with observations by Danforth & de Aberle (1928) upon 500 pregnant mice. They interpret the lack of correlation as the resultant of (1) an initial negative correlation between the number of eggs on the two sides, and (2) heterogeneity between the females in factors affecting pre-implantational loss of eggs, causing a superimposed positive correlation. The negative correlation found by Runner (1951) in his unoperated controls may be due to the use of more homogeneous stocks than those used by Danforth & de Aberle and by ourselves, since his control females were taken from a single inbred strain. On the other hand, Hollander & Strong (1950) also found a negative correlation in 1080 females of very diverse origin.

Transfer of eggs weakens or removes the initial negative correlation between the number of eggs on the two sides, and should therefore result in a positive correlation between the number of implantations in the two horns. Runner found a positive correlation between the number of live embryos in the two horns (data on dead and resorbing embryos were not published) in females to which unfertilized eggs had been transferred. These females were taken from four different inbred strains, and so were probably less homogeneous than his controls. This factor, as we have seen above, would tend to increase the positive correlation.

In our own data we found a large and statistically significant *negative* correlation in recipients of $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ transfers between the number of successful implantations in the two horns ($r = -0·483$, $n = 31$, $P < 0·01$). The greater part of the correlation arose from those females which received large numbers of eggs. In Table 14 the recipients of $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ transfers are divided into those receiving 5–8 eggs and those receiving 9–18 eggs, and are compared with the $0 \rightarrow 2\frac{1}{2}$ transfers and with the unoperated controls. It will be seen that, as the average number of implantations increases, the degree of negative correlation also increases, and is especially pronounced in the group in which the average number of implantations in the left horn was markedly raised above the normal level (transfers of 9–18 eggs).

The simplest interpretation of these results is that the implantation of eggs in one horn hinders the implantation of other eggs, not only in the same horn, but also to some extent in the other horn; but that the process only operates to a

substantial extent when the number of implantations is raised above the normal level. It can be seen from Table 11 that as the number of eggs injected increases, so the number of implantations in the uninjected horn decreases; this trend is statistically significant ($b = -0{\cdot}16$, $P < 0{\cdot}05$). As we have seen, this leads to a 'ceiling' for the average number of successful implantations in the two horns together.

Table 14. *The correlation between the number of implantations in the two horns in various groups of mice arranged according to the average number of implantations ('traumatic' operations exluded)*

Group	No. of eggs injected	No. of pregnancies	Correlation	Average no. of successful implantations		
				Right horn	Left horn	Total
0→2½ (dummy transfers)	0	18	+0·075	3·4	2·6	5·9
Unoperated controls	0	31	−0·025	3·5	3·3	6·8
3½→2½	5–8	15	−0·184	4·3	3·7	8·0
	9–18	16	−0·670	3·3	5·1	8·4

This interpretation, however, fails to account for the contradiction with Runner's result. The contradiction would be largely explained if the hindrance to implantation in the uninjected horn were exerted solely by the *precociously* implanting (i.e. alien) eggs; in Runner's work alien and native eggs were contemporary. However, an analysis of our data by the method of partial correlation (Fisher, 1925–50) showed that implantation in the right (uninjected) horn is hindered to the same extent whether the number of precocious alien implantations in the left horn increases while that of natives in that horn stays constant, or vice versa. In either case the effect is very much more marked when large numbers of eggs are injected.

Since the precocity of our aliens does not account for the contradiction between our results and Runner's, we presume either that his experimental females were more heterogeneous than ours (he drew them from four different inbred strains), or that his mice were such that no 'ceiling' to the average number of implantations was reached in the range tested. Fig. 3 suggests that both factors are operating; the frequency distribution for his data shows a greater spread than ours, and, unlike ours, it is roughly symmetrical, showing no evidence of an upper limit to the number of implantations. It should be remembered that his figures refer to living embryos only.

DISCUSSION

Relative stages of donor and recipient

It is possible to gain the impression from the literature on fertilized egg transfer that the best results are obtained by synchronizing the post-coital stages of donor and recipient. Fekete & Little (1942) found that the 52 hr. → 52 hr. combination was more successful than the 52 → 28 and 52 → 76 combinations. The 52 → 28 combination labours under the special disadvantage that the recipient uterus is required to accommodate eggs 2 days earlier than it would normally receive

them. Beatty (1951) obtained his five successes from more or less synchronous combinations. Both Fekete & Little and Beatty also made transfers of 3-day eggs, but in neither case was the stage of the recipient stated. It is therefore not clear whether or not the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ combination, which we found to be superior to the synchronous $3\frac{1}{2} \rightarrow 3\frac{1}{2}$ and $2\frac{1}{2} \rightarrow 2\frac{1}{2}$ combinations, was also tested by these authors.

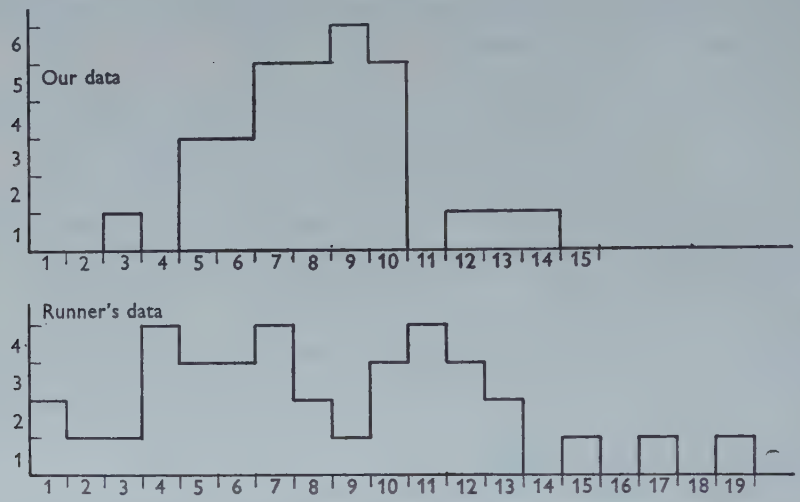

Fig. 3. Frequency distributions, for our data and for Runner's, of the total numbers of implantations and of live embryos respectively in unoperated control females (both horns combined).

There are thus no published data which contradict our finding that, at least when in competition with native eggs, $3\frac{1}{2}$-day eggs transferred to $2\frac{1}{2}$-day recipients give strikingly better results than are obtained from the two synchronous combinations which we tested. Boot & Mühlbock (1953) also tested the 3-day → 2-day combination, and commented that 'None of the other time combinations gave better results'. Unfortunately, they did not reveal what other combinations they had studied, nor did they distinguish results obtained from recipients mated with fertile males from results obtained from pseudo-pregnant recipients which had been mated with vasectomized males. Chang (1950) transferred fertilized rabbit eggs of various ages to pseudo-pregnant recipients of various post-coital stages, and found that, for a given donor stage, the synchronous combination was always as good as the best non-synchronous combination. However, he overstated his findings in his summary, when he said: 'Thus there is little or no chance for a transferred ovum or blastocyst to develop into young when it is in the tracts one or two days before or after the corresponding corpus luteal stage.' His own results, in fact, showed that 2-day → 3-day transfers were as successful as 2 → 2 transfers, 4 → 3 as successful as 4 → 4, and 6 → 5 as successful as 6 → 6.

The viability of artificially ovulated eggs from sexually immature females

In discussing their work on the transfer of unfertilized mouse eggs, Runner & Palm (1953) make the following comment: 'Ova artificially ovulated from pregnant mice and transplanted to recipient foster mothers were shown by one of us (Runner, '51) to have a survival of 20%. The present results based on ova artificially induced from prepuberal mice and transplanted at the time of ovulation provided a survival of 14·2%. Although the two experiments were of somewhat dissimilar design, available data indicate that unfertilized ova from prepuberal donors may survive less frequently than do ova from pregnant donors.' The difference was not in fact statistically significant ($\chi^2 = 0{\cdot}928$, $P > 0{\cdot}3$).

Our results show no difference in viability after transfer at $3\frac{1}{2}$ days between spontaneously ovulated eggs from mature donors and artificially ovulated eggs from immature (30-day-old) donors. Gates (1956) has obtained similar results from transfers of $3\frac{1}{2}$-day eggs to $3\frac{1}{2}$-day recipients.

This result, if generally valid, is important in connexion with agricultural applications of egg transfer. In the most promising of such applications the eggs are obtained by superovulation of immature females. By this method not only can large numbers of eggs be obtained on a single occasion from a single donor, but also the generation interval can be shortened, since it is not necessary to await the onset of puberty—the calf, for example, can be made to ovulate soon after birth. These advantages could be nullified if eggs obtained in this way possessed subnormal viability, and it is encouraging to find that, in the mouse at least, there is no evidence of such an effect.

Limits to implantation and subsequent survival of large numbers of eggs

Our data show no evidence of a limit to the number of implantations which can occur in a single uterine horn of an adult female mouse; but they suggest that when the average number is increased much above normal, higher rates of embryonic mortality supervene. They also indicate a limit to the number of successful implantations *per female*, since increase above a certain level of the number in one horn is compensated by decrease of the number in the other horn. This might appear to conflict with the findings of Engle (1927), who induced superovulation in adult female mice with pituitary implants. On the ninth or tenth day after mating the number of implantations per female was found to range from nineteen to twenty-nine. At this stage of pregnancy all implantations can be counted. We, however, have only been able to count what we have called 'successful implantations', i.e. those which remain alive long enough to leave a recognizable trace at autopsy on the sixteenth day. It is therefore possible that the limit which we observed was not a limit to implantation but was imposed by mortality occurring immediately after implantation, due perhaps to insufficiency of corpora lutea to supply the progesterone requirements of the excessive number of implantations. Parkes (1942) found that the great majority of implanted embryos in superpregnant rabbits (average of eighteen implantations per female) were already dead and

resorbing by the end of the first third of pregnancy, so that the number of live young produced was actually less than in normal pregnancies. Parkes comments: 'The reason why the uterus is unable to support the growth of supernormal numbers of embryos is not clear, but in view of the stage of pregnancy at which regression seems to have taken place... it is unlikely to be primarily of mechanical or metabolic origin.'

Evans & Simpson (1940) reported similar results in rats, which, however, are not fully comparable since the animals used were sexually immature.

We wish to stress that the average number of live young which female mice are capable of bringing to term is not an absolute quantity but varies according to the genetic make-up of the stocks used. A remarkable colony of mice has been described (Hauschka, personal communication) in which the average litter size is 12·6. Environmental circumstances undoubtedly also play a part. For example, Searle (personal communication) has found that embryonic mortality approaches 100% if the females are not allowed to mate until they are 8 months old.

One-horn versus two-horn operations

A point of practical importance on which our results throw light is whether to inject all of the eggs intended for a given recipient into one horn of the uterus, or whether to distribute them equally between the two horns. Our data indicate that, at least so long as the expected number of alien embryos recovered does not exceed about three, nothing is gained in exchange for the time and trouble lost by splitting the inoculum. At what point above this level we would encounter diminishing returns due to overcrowding remains to be determined. Our evidence of increased mortality in the injected horn associated with large inocula suggests that we may have already reached the threshold of diminishing returns. Hollander & Strong (1950) obtained sixty-eight pregnancies after unilateral ovariectomy. This procedure raised the average number of implantations *per horn* to more than eight. They found no significant increase in embryonic mortality. However, our method of crowding a single horn differs from theirs in that the number of embryos carried by the female may exceed the number of her corpora lutea.

Limits to the yield of live young from the transfer of fertilized mouse eggs

The factors limiting the yield obtained from egg transfer are of two kinds: (1) The natural hazards causing loss between mating and parturition in unoperated females of the recipient stock; in operated females, the transferred eggs are presumably as much exposed to these hazards as are the native eggs. (2) The technical hazards arising from the procedure of transfer which cause additional loss of transferred eggs.

(1) *Natural hazards*

These can conveniently be divided into failure, for whatever reason, to become pregnant, and loss by females which become pregnant of some of their eggs between fertilization and birth.

Failure to become pregnant is the easier to eliminate. Our recipient females (including unoperated controls) gave a pregnancy rate of only 60%, but prior to mating they were kept in relatively bad environmental conditions, being stored in large stock cages containing about twenty to thirty mice in each cage. We find that in pair matings of cross-bred mice about 80% of post-partum heats are followed by pregnancy (Michie, 1955), unless mating occurs in every post-partum heat, this underestimates the pregnancy rate. The colony of mice which we mentioned previously as having an exceptionally large average litter size approaches a 100% pregnancy rate.

Things are different with inbred mice. In the C57BL and C3H inbred strains we find the post-partum pregnancy rate to be less than half the rate in inter-strain F_1 hybrid females (Michie, 1955). But where the conditions of the experiment necessitate the use of inbred recipients, other means, which we did not use, are at hand for increasing the pregnancy rate in recipients. First, Fischberg & Beatty (personal communication) report a strong correlation between the size of the vaginal plug and the probability that pregnancy will ensue. Females with small plugs could be rejected. Secondly, all females could be rejected whose ovaries did not show signs of recent ovulation. In our operations only the left ovary was exposed to view, but if this ovary had not ovulated we found that the odds were against the recipient's becoming pregnant.

The natural loss of eggs between fertilization and birth in those females which become pregnant has been estimated by Fekete (1947) as 16% in the C57BL inbred strain and 42% in the DBA inbred strain. This quantity, which is evidently highly dependent upon the genetic make-up of the mice, is compounded of failure of fertilized eggs to implant, and failure of those eggs which implant to survive to birth. In our material at least 14% of all implanted embryos died, as judged by the observed resorption rate. We know of no direct estimate in the mouse of the first component, i.e. the natural loss of fertilized eggs through failure to implant. Danforth & de Aberle (1928) estimated that 24% of corpora lutea in their mice were not represented by implantation sites, but this quantity also included eggs which ripened but were not shed, and eggs which were shed but were not fertilized.

(2) *Technical hazards*

These can be divided into whole-inoculum loss, partial loss of inocula through injury or escape of some of the eggs, and reduction of implantation rate due to surgical interference.

Whole-inoculum losses were estimated in our material as accounting for about 33% of all eggs injected. These losses must arise from technical errors which presumably could be eliminated, although at present we can only guess at their nature. Runner (1951) seems to have encountered a similar phenomenon in transfers of unfertilized eggs to the ovarian capsule; his results, when tabulated in the same way as we have tabulated ours in Fig. 1, suggest a frequency of whole-inoculum loss similar to ours.

Loss through reduction of the implantation rate by surgical interference was found by us to be about 33%. It was considerably greater in operations classified as traumatic. This suggests that the loss could be further reduced by improvement of surgical technique.

We can now make a very rough estimate of the remaining technical hazard—loss through injury or escape of individual eggs—by calculating the expected yield from eggs transferred to recipients which became pregnant, after allowing for the other factors which we have listed. The answer can then be compared with the yield actually obtained.

We assume that 33% of injected eggs were lost as whole inocula, that of the remainder a proportion, say not more than 20%, failed through natural causes to implant, that of the remainder 33% failed to implant owing to surgical injury to the uterus, and that of the remainder 14% died after implantation. This leads to an expected yield of about 30% as compared with 22% actually obtained. Hence injury or escape of parts of inocula probably accounts for about a third of all injected eggs. Here again it is reasonable to hope that technical improvement could reduce this source of loss.

If whole-inoculum loss could be eliminated and loss from the other two technical hazards could be reduced by, say, half, then yields around 50% of all eggs injected could be attained from moderately favourable recipient stocks, even allowing some failures to become pregnant. This represents, perhaps, a reasonable target for the immediate future. The nearest approach to date has been made by Gates (1956) with a yield of 40·5% of 925 eggs injected in transfers from $3\frac{1}{2}$-day donors to 126 $3\frac{1}{2}$-day pregnant recipients.

SUMMARY

1. The origin and potential uses of the method of egg transfer in mammals are briefly surveyed.

2. An experiment is described in which genetically labelled fertilized mouse eggs were transferred to the left uterine horns of recipient female mice. Eggs were obtained both by induced ovulation of sexually immature donors and by spontaneous ovulation of adult donors. Both pregnant and pseudo-pregnant recipients were used. The post-coital stages of donors and recipients were independently varied. At $16\frac{1}{2}$ or $17\frac{1}{2}$ days post-coitum the recipients were killed and their uterine contents recorded.

3. The operation had no effect upon the recipients' chances of becoming pregnant, nor did it have substantial effects upon the implantation and subsequent survival of eggs in the *uninjected* horn of the uterus.

4. In the injected horn, the implantation rate was reduced by about one-third in recipients both at $2\frac{1}{2}$ and $3\frac{1}{2}$ days post-coitum. Post-implantational mortality in the injected horn was increased in $3\frac{1}{2}$-day recipients, but not in $2\frac{1}{2}$-day recipients, except when the operation was accompanied by gross surgical trauma.

5. The yield of live embryos from eggs transferred to recipients which had themselves been mated to fertile males was highest in the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ days combination,

lowest in the $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ days combination, and intermediate in the two synchronous combinations. These differences may in part be attributable to competition between native and transferred eggs. Such competition was shown mainly to occur before, during or shortly after implantation, and to be a property of non-synchronous rather than synchronous donor-recipient combinations. But the differences were in part independent of the presence of competing native eggs, as shown by transfers to recipients mated to sterile males; the yield from the $3\frac{1}{2} \rightarrow 2\frac{1}{2}$ combination was still very much greater than that obtained from the $2\frac{1}{2} \rightarrow 3\frac{1}{2}$ combination.

6. Transfers of eggs artificially ovulated from sexually immature donors gave results in all respects similar to those obtained with eggs spontaneously ovulated by sexually mature donors.

7. The distribution of alien embryos among the recipients suggested that apart from the random loss of parts of inocula through escape or death of individual eggs, there was another and distinct process at work causing the loss of whole inocula as units.

8. Over the range tested (0–18 eggs) the number of alien embryos and the number of implantations of all sorts in the injected horn rose linearly with increasing numbers of eggs injected. The number of native embryos in the injected horn declined with increasing numbers of eggs injected.

9. When the number of implantations, with increasing numbers of eggs injected, began to exceed the normal quota for one horn, the number of live embryos in the injected horn (alien + native) increased less steeply and the proportion of dead and resorbing embryos began to rise.

10. The $3\frac{1}{2}$-day $\rightarrow 2\frac{1}{2}$-day series gave some evidence that when the number of implantations in the injected horn was raised above the normal level, successful implantation in the *uninjected* horn was reduced, so that the total number in the two horns combined never exceeded an average of about $8\frac{1}{2}$.

11. The experimental results are discussed in the light of previous work and of future application. We conclude that with reasonable control of natural and technical hazards a yield of about 50% of fertilized mouse eggs recovered as live young should be attainable.

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PRESSURE RECEPTORS IN THE FINS OF THE DOGFISH *SCYLLIORHINUS CANICULA*

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INTRODUCTION

In 1908 Wunderer described 'terminal nerve corpuscles' (Terminalkörperchen) in the fins of five species of elasmobranch fish, *Mustelus canis*, *Scyllium* (*Scylliorhinus*) *canicula*, *Acanthias vulgaris*, *Centrina salviani* and *Squatina angelus*, and he believed these organs to represent the only sensory structures of this type found in the Anamnia. The organs are situated in the loose connective tissue surrounding the radii and horny rods of the fin skeleton. The horny rods are arranged in two layers, and are separated from the corium of the skin and from each other by connective tissue. Between the two layers of horny rods the connective tissue forms a gelatinous wedge-shaped mass tapering towards the margin of the fin where the two layers of horny threads meet. The fin nerves form a plexus just distal of the end of the cartilaginous skeleton. The plexus gives rise to axially running nerve stems with numerous side branches embedded in the gelatinous connective tissue. The majority of the side branches end in what Wunderer believed to be an encapsulated skein of nerve fibres, myelinated for part of their course through the skein. Dr A. Barets, of the Laboratoire de Biologie Animale of the University of Paris, while working at Plymouth, made methylene-blue preparations of dogfish fins and was able to confirm all essential points of Wunderer's description of the organs. He has kindly let me have one of his preparations, and has given me permission to include a drawing of it in this paper (Fig. 1). The only point in Wunderer's description so far lacking confirmation is the presence of a thin sheath-like capsule surrounding the nerve coil.

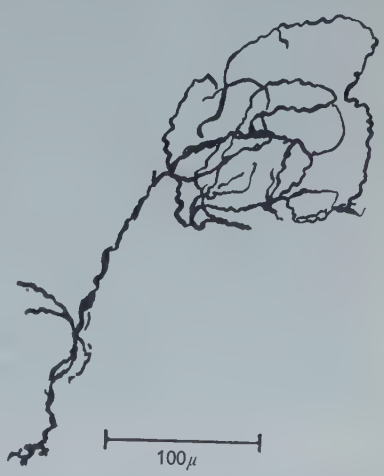

Fig. 1. Terminal corpuscle dissected free from surrounding connective tissue in pectoral fin of *Scylliorhinus canicula*; methylene blue; ×200.

The shape and arrangement of the organs point to their functioning as pressure receptors, and their presence in the fins only, and not, according to Wunderer,

under the skin of head and trunk, made it appear likely that they might act as proprioceptors in the widest sense of the term. The results of the experiments to be described confirm this assumption.

METHODS

Most of the experiments were carried out on the pectoral fin of medium-size dogfish, but identical results were obtained from all the other fins. The fish were killed by decapitation and pithing, and the fins severed from the body. The dorsal and ventral skin was then removed either by stripping it from the muscular part of the fin only, or from the entire surface. Fig. 2 shows a longitudinal section of the

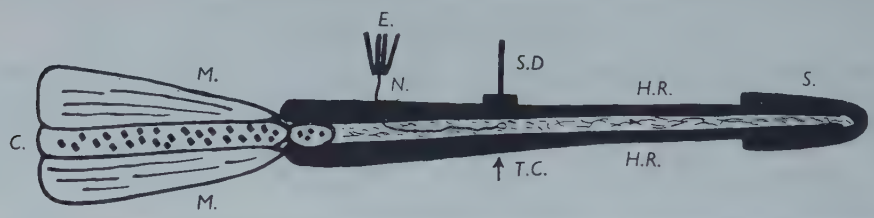

Fig. 2. Diagrammatic longitudinal section through dogfish fin showing position of terminal corpuscle, access to nerve, and mode of application of pressure. *C.* cartilaginous skeleton of fin; *E.* tips of forceps electrode; *H.R.* horny rods; *M.* musculature of fin; *N.* sensory nerve; *S.* remnant of skin; *S.D.* stimulating disc; *T.C.* terminal corpuscle.

fin, and the site at which access was gained to the sensory nerves. A sufficiently long stretch of nerve was isolated in the region distal to the fin musculature and connected to a silver forceps electrode for oscillographic recording; the second electrode was a silver wire making contact with inert tissues of the fin. The fin was mounted flat on a Perspex plate for stimulation by pressure, which was effected by lowering metal disks of various diameters on to the surface of the fin by means of a vertical screw device. Alternatively, only the proximal region of the fin was clamped down to the plate, and the distal portion projecting freely from the holder could be deformed by dorso-ventral bending.

RESULTS

When the preparation is mounted for recording, a certain amount of irregular discharge activity is picked up. This usually consists of spike potentials of a considerable range of amplitude, obviously derived from fibres of a similarly large range of diameters. Fig. 3 A shows a representative record of this kind. It would be wrong, of course, to describe all this as spontaneous activity. As will be seen presently, the adaptation of the terminal corpuscles is slow, and there are bound to be lasting stresses in the mounted fin which may well be responsible for much of the continuous activity of the preparation. It is relatively easy to isolate the impulse discharge from a single functional unit by careful selection of the site of stimulation. The 'adaptation' of a single unit to continued pressure is shown in Fig. 4. The discharge frequency, after having reached its maximum during the application of pressure, falls fairly rapidly to about a third of its maximum value, and then declines more slowly.

Fig. 5 shows a series of single-unit responses to a step-by-step increase in pressure interrupted by periods of 'adaptation'. The higher level of discharge activity at the end of the experiment was recorded for a period of over 40 sec., and was observed

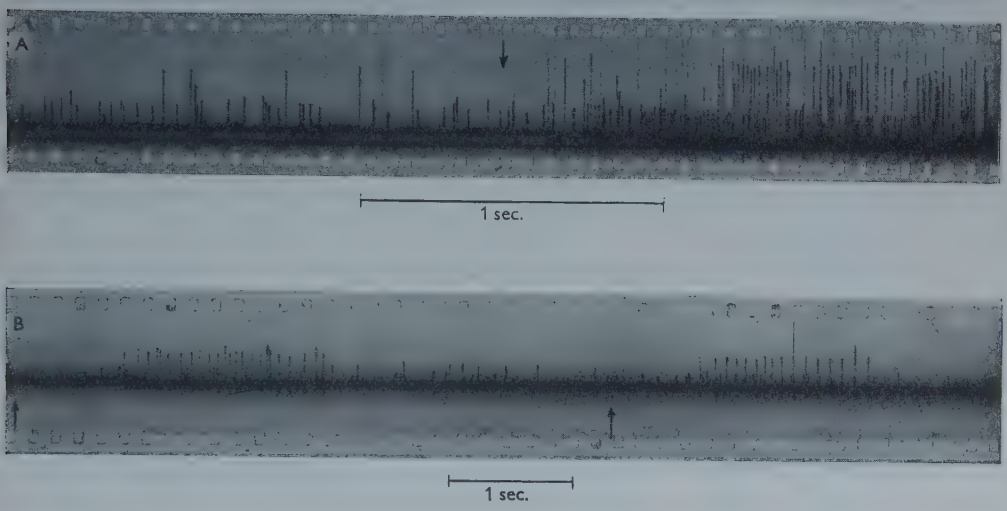

Fig. 3. Records of impulse discharge from terminal corpuscles. A. Multi-unit discharge recorded before and after application of pressure. ↓ marks contact of mechanical stimulator with fin. B. Response to repeated ventral flexion of fin. ↑ marks contact of mechanical stimulator with fin. (Reduced amplification.)

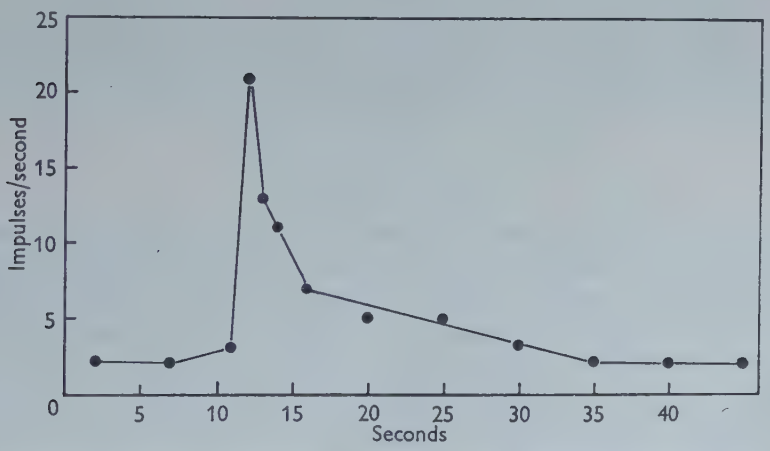

Fig. 4. Single-unit response from terminal corpuscle showing time course of decline in discharge frequency during continued application of pressure.

to go on unaltered for a number of minutes after the end of the recording. The 'adaptive' behaviour shows a considerable latitude, and this is the case even among unit discharges of the same amplitude and speed of propagation. Probably a considerable part of the decline in discharge frequency, especially the initial rapid fall,

may be caused by mechanical accommodation connected very likely with the viscous flow of the gelatinous tissues in which the end-organs are embedded, and it would appear safe to assume that neurologically the organs are of the slowly adapting type. The existence of mechanical accommodation would also explain the observation that successive applications of identical pressures do not as a rule yield quantitatively identical responses, a fact which made a study of the quantitative stimulus/response relationship impossible.

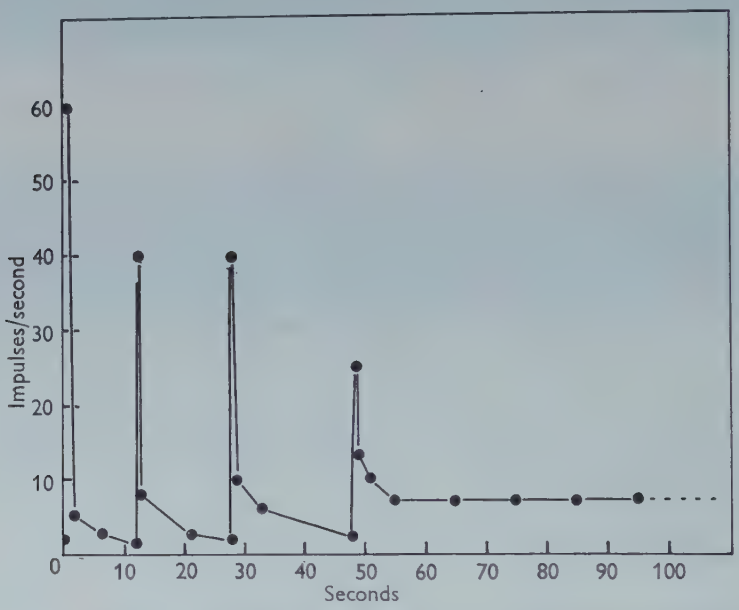

Fig. 5. Single-unit responses from terminal corpuscle to a step-by-step increase in pressure, followed by periods of steady pressure.

DISCUSSION

Wunderer quite rightly diagnosed the sensory nature of the terminal corpuscles, and also correctly described them as pressure receptors. They are by their distribution and behaviour admirably suited to signal the spatio-temporal patterns of pressure changes arising in the course of active and passive deformation of the fins. This is well illustrated by the record of the effect of repeated ventral deflexion of the fin (Fig. 3 B). It may thus be legitimate to consider the organs as proprioceptors in the widest sense of the term.

Muscle receptors of the type described in *Raja* by Polonmordwinoff (1898) and functionally analysed by Fessard & Sand (1937) do not appear to be present in the fins of the dogfish (Barets, personal communication). However, diffuse nerve endings are found in the muscle fasciae, and they are the only sensory structures connected with the fin musculature to which a possible proprioceptive function could be attributed. The fact that the responses described in this paper were picked up far distal of the muscle layer makes it certain that they were definitely not derived

from the latter endings. The degree of contribution of the less highly organized nerve endings in the skin to the control of posture and steering movements of the fin is difficult to assess. On recording from parts of the fin covered with skin, it was found that the discharge activity in any given nerve twig was more complex. Responses from the skin of the clasper, where the absence of terminal corpuscles makes possible the study of skin receptors in isolation, showed that there is a sufficient overlap in impulse characteristics between the impulses from skin receptors and those from Wunderer's organs, to make it difficult to discriminate between them in preparations of the fin covered by intact skin.

SUMMARY

1. The presence of so-called terminal corpuscles in the connective tissues of the fins of *Scylliorhinus canicula*, first described by Wunderer in 1908, is confirmed.

2. It is demonstrated that they are pressure receptors with a slow rate of adaptation.

3. From their topographic distribution and from their mode of response to mechanical stimulation, it is postulated that the terminal corpuscles serve as proprioceptors in the widest sense of the term by signalling the spatio-temporal patterns of active or passive deformation of the fin.

4. Their topographic distribution makes it possible to distinguish their responses from those of sense endings associated with the muscles or tendons of the fin.

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THE INTERACTION BETWEEN METALS AND CHELATING AGENTS IN MAMMALIAN SPERMATOZOA

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Several studies have been made on the bacteriostatic and fungicidal properties of oxine (8-hydroxyquinoline) and other chelating agents, and it has been suggested that they might act, in part at least, by depriving cells of essential trace metals (Albert, 1951; Martell & Calvin, 1952).

In an attempt to determine the trace element requirements of mammalian spermatozoa, White (1955) investigated the effect of several chelating agents on motility. A number were found to be toxic to ram, bull, rabbit and human spermatozoa. In fact some (e.g. sodium diethyl dithiocarbamate, 1-nitroso-2-naphthol and ethyl potassium xanthate) were much more spermicidal than oxine, which has long been used as a chemical contraceptive in the form of Chinasol. Tests with bull semen showed that cobalt reduced the toxicity of sodium diethyl-dithiocarbamate, nitrosonaphthol and *o*-phenanthroline. Other mixtures of heavy metals and chelating agents were, however, more toxic than the chelating agents alone.

Further studies on the interaction between metals and chelating agents in bull, ram, rabbit and human spermatozoa are presented in this paper.

MATERIALS AND METHODS

Semen and diluents

The method of collecting semen was the same as described previously (White, 1954). An isotonic diluent of pH 7·0 was used in all experiments and had the following composition: 0·032 M-$NaH_2PO_4H_2O$, 0·048 M-$Na_2HPO_4.12H_2O$, 0·036 M-NaCl, 0·004 M-KCl, 0·022 M-fructose or glucose. Glucose was only used in the experiments with purified diluents (Tables 3 and 5). The metals referred to in Table 3 are copper, cobalt, manganese, iron and zinc, which were all added as the A.R. salts. The first three were used at a final ionic concentration of 0·05 mg./100 ml. and the last two at a concentration of 0·20 mg./100 ml. The chelating agents were B.D.H. Laboratory reagents.

Motility scoring

The semen was diluted 1 in 40 in small tubes for motility observations which were made at 37° C. Motility was scored at hourly intervals over a 4 hr. period by the system of Emmens (1947). Full motility was rated as 4 and complete immotility

as zero. Quarter grades were frequently used, and in order to eliminate fractions, the total 4 hr. score for each ejaculate has in all cases been multiplied by 4. The highest possible score, based on five observations, is 80.

Statistical analysis

Where responses have been virtually of the all or none type, statistical analysis has not been undertaken. In other cases, experiments have been evaluated by a *t*-test or by the analysis of variance (Fisher, 1955), with isolation of sums of squares attributable to differences between ejaculates and treatments. The total figure for the period has been used as unit observation and the treatment-ejaculate interaction mean square as the error term.

Purification of diluents

Diluents were purified by the absorption method recommended by Donald, Passey & Swaby (1952). Five g. Al_2O_3 (British Drug Houses Ltd. chromatographic absorption analysis material) was added to 500 ml. of diluent in a conical flask and heated with frequent swirling for 1 hr. The flask was then left to cool in a refrigerator over night and filtered through an acid-treated, sintered glass filter connected by glass joints to a Pyrex filter flask.

Test of purity of diluents

The following test for heavy metals is based on the method of Stout & Arnon (1939). Ten millilitres of glass-distilled water, 5 ml. of redistilled chloroform, 10 drops of 0·1% dithiozone in redistilled chloroform and 5 drops of ammonia (redistilled, approximately 6 N) were shaken in a small Pyrex separating funnel. The chloroform layer was run off and 5 ml. more added and shaken. The chloroform (which was found to separate much more rapidly in cold weather on exposure to an infra-red lamp) was now colourless or a slight blue. Ten millilitres of the test solution were then added to the funnel which was shaken and the colour of the chloroform layer noted. The funnel was rinsed with 0·5 N-HCl between determinations to remove traces of metal adhering to the wall (see Piper, 1944). Standard zinc sulphate solutions made up with glass-distilled water gave the following colours: 1·00 μg. Zn/ml., intense red; 0·10 μg. Zn/ml., purple pink; 0·01 μg. Zn/ml., no colour or light blue.

Using this method the following approximate estimates of heavy metal content were obtained: tap water, 1·00 μg./ml.; diluents in either glass-distilled or ordinary distilled water, 0·10 μg./ml.; ordinary distilled water, 0·02 μg./ml.; glass-distilled water, purified ordinary distilled water, purified diluents, 0·01 μg./ml.

RESULTS

Previous tests (White, 1955) showed that cobalt, copper, zinc and cadmium could influence the toxicity of some chelating agents to bull spermatozoa. This observation has been checked for the semen of this species and extended to other spermatozoa. The result of a comparative study on bull, ram, rabbit and human semen is set out

in Table 1. The chelating agents used were nitrosonaphthol, *o*-phenanthroline, sodium diethyldithiocarbamate, cupferron (*N*-nitrosophenylhydroxylamine), ethyl potassium xanthate and hydroxyquinoline, all at 0·1 mM., with metals at 0·2 mM. concentration. All chelating agents have been previously shown to be toxic to mammalian spermatozoa (White, 1955). An overall analysis of variance has not been undertaken since the variances of the groups in Table 1 were obviously heterogeneous. This is due to the extremely spermicidal nature of some of the metal-chelate mixtures, in which the effects of the metals are so clear cut anyway as to make statistical analysis unnecessary. The significance of conclusions concerning the reverse effect of cobalt have, however, been checked by comparing groups A and B by means of the *t*-test.

Table 1. *The effect of* 0·2 mM. *metal ions on the toxicity of* 0·1 mM. *chelating agents for bull, ram, rabbit and human spermatozoa. Values represent the mean total motility score over a* 4 *hr. period at* 37° C.

Chelating agent	Species	Ejaculates	Control	Chelating agent plus following metal				
				A Nil	B Cobalt	C Copper	D Zinc	E Cadmium
1-Nitroso-2-naphthol	Bull	4	69	15	72*	0†	11	9
	Ram	4	73	17	74*	0†	10	1
	Rabbit	4	65	9	66*	0†	6	3
	Human	5	58	5	52*	0†	5	2
o-Phenanthroline	Bull	10	55	23	48*	25	24	30
	Ram	4	74	18	68*	11	22	16
	Rabbit	4	66	7	27‡	8	14	11
	Human	5	59	10	17	8	11	9
Sodium diethyl-dithiocarbamate	Bull	4	51	18	44*	7	3	5
	Ram	4	72	57	60	4†	4†	3†
	Rabbit	4	61	11	22	9	5	0†
	Human	5	54	3	21	7	5	1
Cupferron (*N*-nitrosophenyl-hydroxylamine)	Bull	4	60	21	16	0†	12	23
	Ram	4	76	40	37	0†	42	39
	Rabbit	4	49	38	39	1†	39	33
	Human	4	43	15	15	9	16	10
Ethyl potassium xanthate	Bull	4	48	16	20	2†	11	0†
	Ram	4	77	64	55	0†	28†	0†
	Rabbit	4	48	23	26	0†	17	0†
	Human	4	44	16	17	4†	17	3†
8-Hydroxyquinoline (oxine)	Bull	4	56	29	13	1†	7†	8†
	Ram	4	79	46	26	4†	0†	4†
	Rabbit	4	48	14	16	0†	5	1†
	Human	4	44	5	10	0†	4	3

* Highly significantly better than A, $P > 0{\cdot}01$. † Obviously less than A. ‡ Significantly better than A, $P > 0{\cdot}0$

Cobalt caused a highly significant decrease in the toxicity of nitrosonaphthol for ram ($t = 19{\cdot}0$, $P > 0{\cdot}01$), bull ($t = 11{\cdot}9$, $P > 0{\cdot}01$), rabbit ($t = 9{\cdot}1$, $P > 0{\cdot}01$) and human spermatozoa ($t = 13{\cdot}6$, $P > 0{\cdot}01$). It had a similar effect on *o*-phenanthroline with the ram ($t = 11{\cdot}9$, $P > 0{\cdot}01$), bull ($t = 3{\cdot}0$, $P > 0{\cdot}01$) and rabbit ($t = 3{\cdot}5$, $P > 0{\cdot}05$), and on sodium diethyldithiocarbamate in the case of bull spermatozoa ($t = 6{\cdot}4$, $P > 0{\cdot}01$).

There can be little doubt that, for all species, nitrosonaphthol, ethyl potassium xanthate and hydroxyquinoline are more spermicidal in the presence of copper. Similar effects were seen with cupferron using bull, ram and rabbit spermatozoa and with sodium diethyl dithiocarbamate in the case of the ram.

The toxicity of sodium diethyldithiocarbamate, ethyl potassium xanthate and hydroxyquinoline was clearly greater for ram spermatozoa in the presence of zinc. In the case of the last chelating agent a similar effect was also seen with bull sperm.

Cadmium enhanced the activity of ethyl potassium xanthate for all species and of hydroxyquinoline for the bull, ram and rabbit. Similar effects were seen using sodium diethylthiocarbamate with ram and rabbit spermatozoa and nitrosonaphthol with the former species.

Table 2. *The motility of bull, ram and rabbit spermatozoa in the presence of* 0·1 mM. *chelating agents,* 0·2 mM. *metal and a mixture of the two. Each value is the mean of the total motility score over a* 4 *hr. period for two ejaculates at* 37° C.

Chelating agent	Metal	Species	Motility score			
			Control	Chelating agent	Metal	Chelating agent + metal
Ethyl potassium xanthate	Copper	Bull	68	38	65	7
	Zinc	Ram	70	40	73	14
	Cadmium	Rabbit	52	17	62	0
8-Hydroxyquinoline (oxine)	Copper	Bull	60	43	63	1
	Zinc	Ram	67	62	69	0
	Cadmium	Rabbit	53	13	66	2
1-Nitroso-2-naphthol	Copper	Bull	69	14	67	0
Cupferron (*N*-nitrosophenyl-hydroxylamine)	Copper	Bull	68	19	67	0
Sodium diethyl-dithiocarbamate	Zinc	Ram	67	47	72	0

The next experiments were designed to test if the increased toxicity of the metal-chelate mixtures was due to an interaction between the two or merely to the additive effect of two spermicidal substances. Factorial experiments were done with two bull, ram and rabbit ejaculates using copper, zinc and cadmium respectively and the chelating agents which gave the maximum effect for the particular metal and species in Table 1. The results are set out in Table 2. Direct factorial analysis is not possible because of the very low variance of the highly spermicidal metal chelate mixture which in many cases gave zero motility scores. By inspection, however, it is obvious that the metals were not themselves toxic and that the greatly increased spermicidal activity of the metal-chelate mixture is due to an interaction between the two—in a statistical sense, at least.

Experiments were then undertaken to see if the toxicity of the chelating agents could be decreased by purification of the diluent. In initial tests it was found that the motility of ram and bull spermatozoa was very much reduced in the purified

fructose diluent without the addition of chelating agents. This was probably caused by the thermal decomposition of the ketosugar to toxic products. It was certainly not due to the spermatozoa being deprived of essential trace metals, since the addition of such metals in concentrations normally found in ram seminal plasma (copper, cobalt, manganese 0·05 mg./100 ml.; iron, zinc 0·20 mg./100 ml.) did not improve motility to any extent in the purified diluent. If glucose, which is less readily decomposed by heat, was used instead of fructose then there was no depression of motility on purification of the diluent. These effects are seen in Table 3 which are the results of a factorial experiment involving four bull and four

Table 3. *The effect of purified fructose and glucose diluents on the total 4 hr. motility score of bull and ram spermatozoa at 37° C. in the presence and absence of metal ions*

Species	Ejaculate	Unpurified				Purified			
		Fructose diluent		Glucose diluent		Fructose diluent		Glucose diluent	
		No metals	Added metals	No metals	Added metals	No metals	Added metals	No metals	Added metals
Bull	1	72	62	73	70	58	38	76	76
	2	64	59	63	61	25	28	76	68
	3	47	42	38	40	22	20	34	35
	4	62	60	57	61	21	19	49	51
	Mean	61	56	58	58	32	26	59	58
Ram	1	68	74	71	74	43	41	70	69
	2	70	66	66	73	38	31	54	70
	3	80	78	76	80	48	45	57	78
	4	80	80	72	80	40	49	70	77
	Mean	75	75	71	77	42	42	63	74

ram ejaculates. The analyses of variance (Table 4) reveal a highly significant diluent/purification interaction for the bull and ram. This is due to the toxicity of the purified fructose diluent and accounts for the significant main effects of the diluents and purification. The significant diluent/metal interaction with the ram

Table 4. *Summary of the analyses of variance for the data in Table 5 showing variance ratios with the interaction mean square in italics at the base of the columns*

Source of variation	Degrees of freedom	Variance ratio			
		Bull		Ram	
Between treatments:	7	14·21**		53·69**	
Between diluents	1		30·92**		82·88**
Effect of purification	1		32·57**		185·31**
Effect of metals	1		1·30		7·50*
D/P interaction	1		33·68**		89·44**
D/M interaction	1		0·91		9·06**
P/M interaction	1		0·02		0·63
D/P/M interaction	1		0·02		1·13
Between ejaculates	3	25·67**		10·50	
Residual	21	*53*		*16*	

* $P<0·05$. ** $P<0·01$.

is due to the slightly improved motility on the addition of the metals to the glucose diluent. The effect, however, is very small and probably unimportant. Table 5 shows the results of toxicity tests of chelating agents for bull, ram, rabbit and human spermatozoa in purified and unpurified glucose diluent. There is no evidence that the toxicity of the chelating agents is affected by purification.

Table 5. *The toxicity of some chelating agents for bull, ram, rabbit and human spermatozoa in purified and unpurified diluents. Values represent the mean total motility score over a 4 hr. period at 37° C. for five bull, four ram, six rabbit and four human ejaculates*

Chelating agent	Bull		Ram		Rabbit		Human	
	Unpurified diluent	Purified diluent	Unpurified diluent	Purified diluent	Unpurified diluent	Purified diluent	Unpurified diluent	Purified diluent
Nil	49	47	48	48	44	39	55	56
1-Nitroso-2-naphthol	13	10	14	13	17	11	7	14
o-phenanthroline	21	17	19	19	17	17	38	26
Sodium diethyldithiocarbamate	12	6	32	15	5	4	0	0
Cupferron (*N*-nitrosophenylhydroxylamine)	27	17	19	22	15	15	20	17
Ethyl potassium xanthate	33	22	48	41	9	19	9	6
8-Hydroxyquinoline (oxine)	24	29	33	27	8	9	8	11

Nickel combines more readily than cobalt with most chelating agents (Williams, 1953). If cobalt acts by competing with other heavy metals to form a non-toxic complex, then nickel might be expected to be equally effective, provided, of course, the complex formed is also non-toxic. This possibility and also the action of vitamin B_{12} were examined in the next experiment (Table 6) in which the effect was studied of 0·2 mM. nickel, 40 μg./100 ml. vitamin B_{12} and 0·2 mM. cobalt on

Table 6. *A comparison of the effect of* 0·2 mM. *nickel*, 40 μg. % *vitamin* B_{12} *and* 0·2 mM. *cobalt on the toxicity of* 0·1 mM. *nitrosonaphthol and* 0·1 mM. *o-phenanthroline for bull and ram spermatozoa. Values represent the mean total motility score over a 4 hr. period at 37° C.*

Chelating agent	Species	Ejaculates	Motility scores				
			A Control	B Chelating agent	C Chelating agent + cobalt	D Chelating agent + nickel	E Chelating agent + vitamin B_{12}
o-Phenanthroline	Ram	4	64	17	61*	48*	19
	Bull	5	37	8	25*	29*	9
1-Nitroso-2-naphthol	Ram	4	58	11	57*	1	10
	Bull	4	55	11	62*	4	13

* Highly significantly better than A.

the toxicity of 0·1 mM. nitrosonaphthol and 0·1 mM. *o*-phenanthroline for bull and ram spermatozoa. On testing against group B, cobalt is seen to reduce significantly the toxicity of both *o*-phenanthroline ($t = 18{\cdot}8$ and $8{\cdot}5$ respectively, $P > 0{\cdot}01$) and nitrosonaphthol ($t = 15{\cdot}9$ and $18{\cdot}9$ respectively, $P > 0{\cdot}01$) for ram and bull spermatozoa. Nickel had a similar effect with *o*-phenanthroline ($t = 10{\cdot}0$ and $4{\cdot}9$, $P > 0{\cdot}01$) but the nickel-nitrosonaphthol complex is apparently more toxic than the free chelating agent. Vitamin B_{12} was not effective in reducing the toxicity of either chelating agent.

DISCUSSION

The potentiation of the toxicity of certain chelating agents by copper, zinc and cadmium and its reduction by cobalt seems to be of fairly general occurrence with mammalian spermatozoa. There are, however, some differences in detail between the species. Similar effects have been noted in other biological systems involving chelating agents and metals. Thus oxine is more toxic to fungi (Mason, 1948; Anderson & Swaby, 1951) and certain bacteria in the presence of copper. In the latter instance it has also been possible to reduce toxicity by cobalt (Rubbo, Albert & Gibson, 1950; Albert, Gibson & Rubbo, 1953). MacLeod (1952), too, has found that the bacteriostatic properties of *o*-phenanthroline were increased by copper and decreased by cobalt, nickel and iron. Mixtures of the heavy metals and other chelating agents used here would seem to merit trial as contraceptives and possibly also as fungicidal and bacteriostatic agents. Invertebrate spermatozoa differ from mammalian spermatozoa in that chelating agents are not toxic to them in the concentrations used here nor is there any potentiation of toxicity by heavy metals. In fact, certain chelating agents may reduce the toxicity of copper and other metals to diluted sea-urchin spermatozoa (Rothschild, 1950; Tyler, 1953; Rothschild & Tyler, 1954).

In the complete absence of copper or iron, oxine has been shown to be completely innocuous to *Aspergillis niger* (Anderson & Swaby, 1951) and Gram-positive bacteria (Rubbo *et al.* 1950; Albert *et al.* 1953). It is tempting to suggest that the spermicidal action of the chelating agent used here might also be normally dependent upon combination with traces of metals such as copper. The reverse effect of cobalt could then be due to it competing with these metals and thus preventing the formation of spermicidal complexes. The fact that nickel, which is higher than cobalt in the Mellor-Maley series (see Albert, 1951), can replace cobalt in the case of *o*-phenanthroline is consistent with the hypothesis that both metals act in this way. On the other hand, there was no evidence in these experiments that the toxicity of the chelating agent is reduced by purification of the diluent. The possibility cannot be excluded, however, that the chelating agents themselves contained traces of metals. It is unlikely that the free metal content of most mammalian semen would be sufficient to mask the effect of purifying the diluent. The inability of vitamin B_{12} to replace cobalt in reducing the spermicidal activity of *o*-phenanthroline and nitrosonaphthol would suggest, as might be expected, that their toxicity is not concerned with the inactivation of this substance.

It should also be borne in mind that the oxidation products of at least some of the chelating agents, e.g. sodium diethyldithiocarbamate and potassium ethyl xanthate, might be the final toxic agents (Keilin & Hartree, 1940). If this is so, then copper and other metals might act by influencing the rate of their formation.

SUMMARY

Studies have been made over a 4 hr. period at room temperature on the effect of 0·1 mM. chelating agents and 0·2 mM. metals on the motility of bull, ram, rabbit and human spermatozoa.

Cobalt decreased the toxicity of (1) nitrosonaphthol for all four species, (2) *o*-phenanthroline for the first three and (3) sodium diethyldithiocarbamate for the bull.

Copper increased the toxicity of (1) nitrosonaphthol, ethyl potassium xanthate and hydroxyquinoline for all species, (2) cupferron for the first three and (3) sodium diethyldithiocarbamate for the ram.

Zinc increased the toxicity of (1) sodium diethyldithiocarbamate, ethyl potassium xanthate and hydroxyquinoline for ram spermatozoa and (2) hydroxyquinoline for bull spermatozoa.

Cadmium increased the toxicity of (1) ethyl potassium xanthate for all species, (2) hydroxyquinoline for the first three, (3) sodium diethyldithiocarbamate for the ram and rabbit and (4) nitrosonaphthol for the ram.

Factorial experiments using bull, ram and rabbit spermatozoa indicated that the spermicidal activity of the metal-chelate mixtures were due to an interaction between the two and not merely additive effects of two spermicidal substances.

Purification of the diluent did not influence the toxicity of the chelating agents to bull, ram or rabbit spermatozoa.

Vitamin B_{12} (40μg. %) did not reduce the toxicity of *o*-phenanthroline or nitrosonaphthol for bull or ram spermatozoa.

Nickel was almost as effective as cobalt in reducing the toxicity of *o*-phenanthroline for ram and bull spermatozoa.

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SOME ASPECTS OF OSMOTIC REGULATION IN LAMPREYS

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I. INTRODUCTION AND METHODS

Although we have some information on osmotic conditions in the migratory lampreys *Lampetra fluviatilis* and *Petromyzon marinus*, no observations on the purely fresh-water form *L. planeri* appear to have been recorded. The main purpose of the work reported in this paper was to study the problem of osmotic regulation in fresh water and most of the observations have been made on adults and ammocoetes of *L. planeri* taken from streams in Somerset. In some instances, however, it has been possible to make comparisons with adults of *L. fluviatilis* fished from the River Dee in October and kept in tap water in the laboratory until the following spring.

Osmolar concentrations were determined by the vapour-pressure method, using a Baldes thermocouple and Zernicke moving-coil galvanometer, and the results have been expressed throughout in terms of millimolar NaCl solution. To facilitate comparison, freezing-point data from other workers have been recalculated in the same terms.

For the Cl analyses of body fluids and tissues extracts volumes of the order of 0·005 ml. were taken up in a fine capillary mounted against a scale. These were then delivered into small silica crucibles and standard $AgNO_3$ solution, made up in 80% HNO_3, was added to them in known volume from an Agla syringe pipette. After standing in the dark for some hours, ether and a few crystals of finely powdered ferric alum indicator were added and titration was then carried out in the same vessel, using N/50 or N/100 KCNS solution delivered from the syringe pipette. The concentration of Cl was then determined by drawing up to the same mark on the capillary a standard solution of NaCl of similar concentration to the unknown and repeating the titration as for the test sample. Using these methods Cl concentration may be determined within 1–2%.

Water content was determined on whole animals, dried in an oven at 105° C. for 24 hr. The finely powdered dried material was then used for fat estimation by extracting it with several changes of ether, followed by petroleum ether. For the determination of total Cl on the dried tissues it was found satisfactory to digest the material with N/30-H_2SO_4 in sealed 5 ml. ampoules placed in an oven at 100° C. for 24 hr. 2 ml. of the dilute acid were added to each sample of about 100 mg., and the subsequent titrations with $AgNO_3$ and KCNS were carried out on aliquot portions of this fluid. Because of the variations in the fat content of the dried

material it was found necessary to express both water and Cl content in terms of the fat-free tissues.

Determinations of osmolar and Cl concentrations were usually made on serum, but in adult *planeri* it was often difficult to obtain sufficient blood for both determinations, and for this reason the peritoneal fluid was sometimes employed. Double determinations on the serum and peritoneal fluid of six animals showed good agreement in total concentration, but there was a tendency for the Cl concentration to be slightly higher in the peritoneal fluid. Where muscle extracts were used, these were prepared by grinding up the tissue in a mortar with quartz sand followed by centrifuging to obtain a clear solution. Determinations were carried out within 20 min. of preparation.

In the experiments involving weight changes the animals were folded gently into a soft absorbent cloth and afterwards weighed in water. Tests on animals which had been wiped off more roughly showed no evidence of increased permeability to water, neither did they appear to lose Cl at a greater rate than animals not subjected to such treatment. For these reasons it is not thought likely that the procedure normally employed would cause any serious skin damage. The accuracy of these weighings may be estimated as about 0·2% for *planeri* and rather less for *fluviatilis*.

In the work on the uptake and loss of chloride, water analyses were carried out by the methods described by Krogh (1937*a*), involving the concentration of a 50 ml. sample to about 0·5 ml. and the subsequent titration of the residue by a modified Volhard method. Although the uptake of the Cl ion may well be secondary to the uptake of Na (Ussing, 1949), Cl determinations alone have been made throughout to simplify the analytical procedures.

Where animals have been depleted of Cl by washing out in distilled water, the volumes used have always been large in relation to the size of the animal (not less than 30 ml./g.), and this water was changed every day. The distilled water was obtained fresh from a large hard glass still and, at least with the analytical methods employed here, contained no detectable quantities of Cl.

II. OSMOTIC CONDITIONS IN NORMAL ANIMALS

(*a*) *Water and fat content*

It became apparent at the outset that, while there were considerable differences in water content between adults of *planeri* and *fluviatilis*, between the ammocoete and adult of *planeri*, and even between ammocoetes caught at different times of the year, these differences were, to a great extent, attributable to variations in the fat content of the dried material. When this was discounted by calculating the water content as a percentage of the fat-free tissues both the range of variation within the various groups and the differences in the mean values were reduced (Table 1). Nevertheless, for *planeri*, fat-free values in the adult appear to be significantly higher than in the ammocoete, and in *fluviatilis* there are indications of an increase in the water content of the body during the period between November and March.

Table 1. *Water and fat content of* Lampetra planeri *and* L. fluviatilis. *The standard errors are quoted after the mean values, and the figures in brackets indicate the number of animals on which observations were made*

	Water (% fresh wt.)	Neutral fat (% dry wt.)	Water (% fat-free fresh wt.)
L. planeri			
Ammocoete (June)	78·5 ± 0·2 (24)	32·0 ± 1·4 (24)	84·8 + 0·1 (24)
Ammocoete (Mar.)	84·1 ± 0·2 (24)	5·9 ± 1·3 (24)	84·9 ± 0·1 (24)
Adult (Apr.)	86·0 ± 0·5 (16)	8·5 ± 2·1 (16)	86·8 ± 0·6 (16)
L. fluviatilis			
Adult (Nov.)	77·0 ± 1·0 (8)	31·6 ± 1·7 (8)	82·7 ± 0·7 (8)
Adult (Mar.)	83·2 ± 1·8 (6)	15·0 ± 3·3 (6)	85·5 ± 1·2 (6)

Ammocoetes kept for long periods in tap water have given higher values than freshly caught animals, and there seems little doubt that the raised water content of the sexually mature adult is associated with the prolonged starvation which precedes spawning. Fat content, especially in *planeri*, is very variable, and in the ammocoete there are marked local and seasonal variations. Fig. 1 records the observations made on ammocoetes collected at intervals throughout the year within a stretch of about half a mile in a small stream. In spite of the very great scatter of the individual observations the general trend is clear—a rapid rise in fat content during the spring from a minimum mean value of 5·5% in mid-March to the maximum value of 36% in the middle of May. This period also coincides with the period of maximum growth in length of the ammocoete (Hardisty, unpublished) and is no doubt associated with a spring peak in the algal plankton. During the summer there appears to be little further fat accumulation, and, indeed, in August and September values were somewhat lower than those recorded in May, June and July.

For adult *planeri* very low fat values have been recorded towards the end of the spawning season and particularly in spent animals. On the other hand, values for animals caught in late March and early April before spawning were similar to those observed in adults of *fluviatilis* in March.

(*b*) *Osmolar concentration and* Cl *concentration of body fluids and muscle extracts*

Mean values for the osmolar concentration of the serum are similar in both ammocoete and adult *planeri*, although in both the observations cover a wide range of individual values (Table 2). The higher values found for the serum of *fluviatilis* are similar to those recorded by Galloway (1933) and Deckhuyzen (1904) for this species. Determinations on six animals in March gave slightly lower values than those obtained in November.

The mean chloride concentration of serum and peritoneal fluid from adult *planeri* is nearly twice that of the ammocoete and accounts for no less than 89% of the total concentration. For *fluviatilis* in November the mean Cl concentration represents only 79% of the osmolar concentration, but in the animals killed in March this ratio was increased to a value much closer to that of the adult *planeri* (85%). These Cl values, although in good agreement with those of Galloway

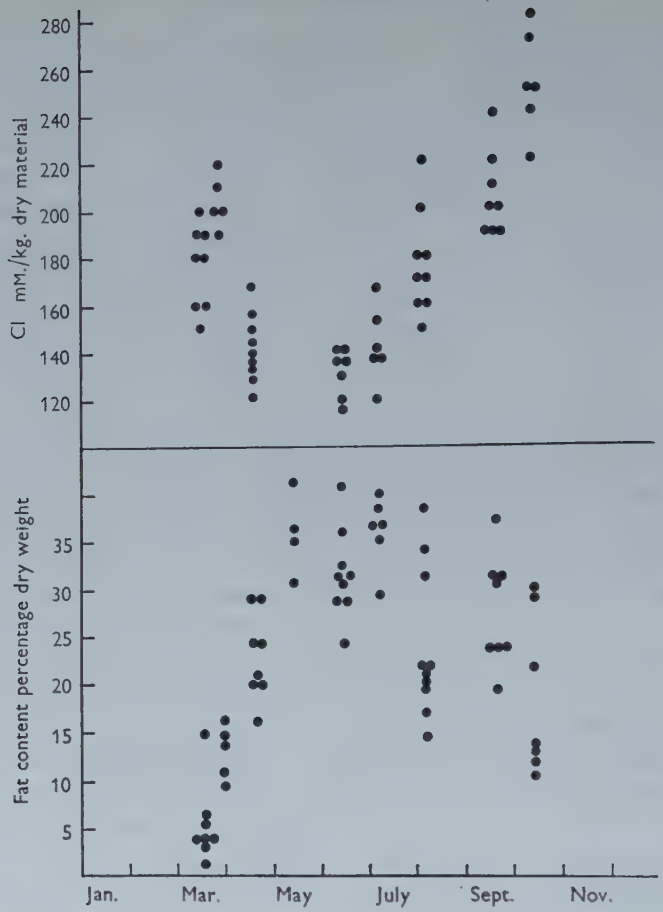

Fig. 1. Seasonal variations in the fat and chloride content of ammocoetes. The values for October are for animals kept for 21 days in tap water (p. 435 and Table 3).

Table 2. *Osmolar concentration,* Cl *concentration and total* Cl *content of ammocoetes and adult lampreys*

	Osmolar concentration (mM. NaCl/l.)	Cl concentration (mM./l.)		Cl conc. / Osmolar conc. %	Total Cl content	
	Serum	Serum	Muscle extract		mM./kg. dry tissues	mM./kg. boc water
L. planeri:						
Ammocoete (June)	110±1·5 (8)	58±0·4 (12)	30±0·9 (6)	53	132±3·7 (24)	24
Adult (Apr.)	113±3·2 (14)	101±1·7 (29)	52±3·2 (6)	89	360±4·7 (8)	55
L. fluviatilis:						
Adult (Nov.)	143±2·5 (6)	113±1·9 (6)	32 (2)	79	210±11·8 (6)	43
Adult (Mar.)	136±3·2 (6)	118±3·5 (6)	—	87	308±25·0 (6)	55
P. marinus:						
Adult (Apr.) (Fontaine, 1930)	158±2·5 (6)	119±0·7 (4)	—	77	—	—

The figures in brackets indicate the number of animals on which the mean values are based.

(1933), are considerably greater than Robertson's (1954) values for the serum Cl of this animal in February. However, it is noteworthy that the total ionic concentration of Robertson's animals suggested a lower level of osmolar concentration than that observed here. In *Petromyzon marinus*, which ascends the rivers in the spring, the osmolar concentration appears to be distinctly higher than in *fluviatilis*, although the Cl fraction forms a similar percentage of the total concentration.

Determinations of the total Cl content of the dried tissues of whole animals after fat extraction indicate that, in *planeri*, the mean Cl content of the adult is more than twice that of the ammocoete taken from the stream in spring and early summer. Assuming all the Cl to be osmotically active and expressing the results as mM./kg. of body water, the overall concentration of this ion in the adult works out at about twice that of the ammocoete, a result which agrees quite well with the direct determinations of the Cl concentration of serum and muscle extracts. Values obtained for *fluviatilis* in November were lower than in the adult of *planeri*, but considerably higher figures were recorded for animals killed in March. These observations, taken in conjunction with the information on Cl concentration, suggest that in both species starvation is accompanied by an increase in the Cl content of the animals. This has been confirmed for the ammocoete by keeping animals, caught in September, for a period of 3 weeks in tap water in the laboratory and comparing their Cl content with that of control animals which had been taken from the stream at the same time and killed immediately (Table 3). In spite of the

Table 3. *Mean water, fat and* Cl *content of eighteen ammocoetes kept in tap water for 3 weeks, compared with a control group of freshly caught animals*

	Fat-free water content as % of fresh wt.	Fat as % of dry wt.	Cl content as mM./kg. fat-free dry tissues
Control animals	86·2 ± 0·03	27·8 + 2·1	200 + 3·6
Animals starved in tap water	86·9 ± 0·04	18·6 ± 3·1	258 ± 8·4

slightly raised water content which has been noted as a normal accompaniment of starvation, these Cl figures for the fasting ammocoetes indicate an overall Cl concentration of 37 mM./kg. body water as compared with 33 mM. for the control group. A marked increase in blood Cl has, in fact, been observed in ammocoetes kept for much longer periods in tap water. Thus three large animals after 2 months starvation gave values of 78, 84 and 86 mM. Cl, and a further specimen kept in the laboratory for 6 months gave 88 mM.; figures which approach the lower range of adult values.

Seasonal changes in the total Cl content of ammocoetes have been followed in those animals which were collected throughout the year for fat determinations. All the animals used were between 70 and 100 mm. long, and, although they were collected from a restricted stretch of the stream, the range of variation in Cl content is considerable. Values plotted in Fig. 1 are for the pooled material from two to four animals. The extent of the seasonal changes in Cl levels was as much as 60%,

with maximum mean values of 200 and 210 mM. Cl/kg. dry material in March and September and minimum values of 130–140 mM. for animals collected in April, June and early July. Taking into account the changes in water content which also occur, the corresponding overall Cl concentrations would fall from a maximum value of 37 mM. Cl/kg. body water in mid-March to a minimum of 24mM. in June. It would be interesting to know whether significant seasonal variations occur also in the Cl concentration of the ammocoete serum. Although examination of the individual observations failed to reveal any close negative correlation between fat content and Cl levels, the general trend of the seasonal changes is suggestive of some relationship between nutritional state and Cl content. Minimum Cl values were observed in early summer when the fat content of the animal had reached its peak and the period of most rapid fat accumulation in early spring coincides with a drop in Cl levels. During the late summer and autumn the spread of the fat values appears to increase with perhaps a slight downward trend, and throughout this period Cl figures show a marked increase. The drop in Cl content in the spring may reflect a displacement of inorganic by organic metabolites in the body fluids, as a consequence of the abundant supply of food available at this time, but it is more difficult to understand why Cl levels should rise steeply later in the summer when feeding is presumably still quite active.

In both ammocoete and adult muscle extracts the Cl concentration is approximately half that observed in the serum, and the values observed agree quite well with those calculated from the Cl determinations made on the dried tissues. Although determinations of the osmolar concentration of muscle extracts have been carried out, little importance is attached to the absolute values in view of the observations of McCormack (1953) on the rapid increase in total concentration of tissue extracts maintained at 0° C. It may, however, be worth recording that the values obtained for ammocoete muscle extracts (mean 144 mM.) were much lower than for the muscle of the adult *planeri* or *fluviatilis* (mean 199 mM.), and that these differences are not accounted for entirely by differences in the Cl concentration of these extracts.

III. THE CHLORIDE BALANCE

(*a*) *Chloride losses and their effects on the concentration of the body fluids*

Observations on the rate of loss of Cl ions from adult *planeri* kept in distilled water showed great individual variations. For the fifteen animals tested, total losses over periods of 4–8 days varied from 9 to 85 μM. Cl, representing daily rates of 1–20 μM. For nine of the experimental animals, however, the daily rate was below 3 μM. and for seven animals which were previously weighed the mean daily rate of loss/g. of body wt. was 1·8 μM. Taking the mean Cl content of the fresh adult tissues as 49 μM./g., this implies a daily loss of nearly 4% of the total Cl in the body, and yet in spite of this rate of loss adults have survived in distilled water for 14 days and ammocoetes up to 21 days. Tests carried out on twelve ammocoetes gave rates varying from 0·2 to 1·2 μM./g./day, and it may well be that a somewhat lower rate of Cl depletion is responsible for the greater tolerance shown by the ammocoete

to distilled water. The highest rates of loss of Cl were generally observed during the first day, reaching minimum values by the third or fourth day and remaining at a low level for the rest of the period.

The effects of Cl depletion on the concentration of the peritoneal fluid were investigated by making Cl determinations on six adult *planeri* before and after washing out in distilled water. At the same time actual Cl losses were measured by Cl analyses of the distilled water (Table 4). Knowing the weight of these animals it was possible to estimate the overall effect of the Cl losses, assuming that the Cl ion is taken equally from body fluids and tissues. Except in one instance, however, the observed decrease in concentration of the peritoneal fluid was considerably greater than the calculated value, implying a relatively greater rate of loss of Cl from the body fluids as compared with the tissues. In spite of the serious reductions in concentration which were observed in these animals, all except one survived the period of washing out. The exception was the animal which had shown an abnormally high rate of loss (51 μM.) and a 50% reduction in Cl concentration. This animal died during the fourth day in distilled water.

Table 4. *Total losses of* Cl *by six adult* planeri *and their effects on the* Cl *concentration of the peritoneal fluid*

Initial Cl concentration (mM. Cl/l.)	Cl concentration after 7 days in distilled water (mM. Cl/l.)	% reduction in concentration	Total Cl losses (μM. Cl)	Calculated % reduction in Cl concentration
117	79	33	42	15
132	86	35	39	7
116	93	19	23	10
104	93	11	22	10
97	48	51	51	28
86	69	20	9	4

Cl estimations have also been carried out on ammocoetes after 14 days washing out in distilled water (Table 5), and the results show a fall in concentration of the same order as that calculated from the known rates of loss of Cl for ammocoetes. The mean percentage reduction in Cl concentration (10%) was less than that observed in the adults which had been washed out in distilled water for 7 days.

Since Cl accounts for nearly 90% of the osmolar concentration of the adult body fluids, the drop in Cl concentration observed in distilled water must inevitably produce a comparable reduction in the total concentration, and indeed decreases of 18 and 29% have been observed in the osmolar concentration of the peritoneal fluid of two adults which had been washed out for 7 days. However, serious as these losses are in their effects on internal concentration, it does not appear that in regard to Cl retentivity *planeri* compares unfavourably with those freshwater teleosts for which similar information is available. Thus for *Ameirus* and *Gasterosteus aculeatus* Krogh (1937*a*) gives figures which indicate rates of loss of about 5 μM./g./day, while even the eel (Callamand, 1943; Krogh, 1939), whose ability to resist starvation in fresh water seems to depend entirely on Cl retention, may lose from

0·2 to 1·8 μM./g./day. For amphibia, Krogh (1937*b*) found rates of 0·7–3·0 μM./g./day in *Rana esculenta*, and for *Amblystoma* kept in millimolar NaCl solutions Jørgensen, Levi & Ussing (1947) reported mean rates of 4 μM./g./day. In making such comparisons, however, it must be borne in mind, particularly where animals are known to have a well-developed capacity for active uptake of Cl ions, that such figures may only represent the net rate of Cl loss, i.e. the resultant of loss and uptake of Cl occurring simultaneously. Nevertheless, with the volumes of distilled water employed here it does not seem likely that active uptake could very seriously reduce the concentration of the water, and it is thought unlikely that the absolute rate of Cl loss is of a higher order altogether than the net rates observed. Wikgren (1953) estimated the absolute rate of loss of *fluviatilis* as about 12–24 μM./g./day, of which only about 1% is lost in the urine. In these experiments, which were generally of short duration, the animals were kept in a smaller volume of distilled water (less than 10 ml./g. of animal), and loss and uptake of Cl were followed by making frequent analyses on the same water. Taking the mean Cl content of *fluviatilis* from Table 2, such losses would imply a depletion of about 70% of the total Cl in the body in 24 hr. It is hard to believe that a similar rate of Cl depletion can obtain in *planeri* in view of the tolerance shown to distilled water over long periods. In the course of the experiment summarized in Table 5, eighteen ammocoetes with a total weight of about 50 g. were kept in a vessel containing 5 l. of freshly distilled water, which was kept running at a rate of approximately 200 ml./hr. Even if uptake could take place under such circumstances it is hardly conceivable that it would substantially reduce the apparent rate of Cl depletion, yet after 14 days all the animals were quite normal and the subsequent analyses of serum Cl showed a relatively small decrease in concentration.

(*b*) *The active uptake of* Cl

Cl absorbing mechanisms are, as might be expected, well developed in both ammocoete and adult. After washing out in distilled water larger animals of 4–5 g. have reduced the Cl concentration of 100 ml. of tap water (0·3 mM. Cl/l.) to about 0·06 mM. after 1 day, and one adult, which during 8 days in distilled water had lost 10 μM. Cl, absorbed this ion from tap water diluted with 5 vol. of distilled water. Some evidence was also obtained of slight absorption from tap water diluted with 10 vol. of distilled water (approx. 0·03 mM. Cl/l.), but at these dilutions Cl analysis becomes uncertain.

For six adults, rates of absorption for the first day in tap water varied from 5 to 30 μM. and for fifteen large ammocoetes from 15 to 30 μM. The corresponding rates per g. were for the adults 5 μM. and for the ammocoete 9 μM., figures which are strikingly similar to those found by Krogh (1939) in *Salmo irideus* and *Leuciscus rutilus*, i.e. 7 and 9 μM./g./day. For *fluviatilis* Wikgren (1953) reports the rate of relative absorption as about 21 μM./g./day, which is much higher than the maximum rate observed in *planeri*.

After 7–14 days in distilled water, absorption in tap water usually continued intermittently for several days, punctuated by periods of pronounced Cl losses.

For eight ammocoetes of 3–6 g. the total net uptake of Cl during 14 days in tap water, following a similar period in distilled water, varied from 48 to 112 μM. with a mean of 71 μM. During the same period net losses to the tap water varied from 12 to 59 μM. with a mean of 36 μM.

There does not appear to be any close quantitative relationship between total Cl uptake and the extent of the previous Cl depletion during the period in distilled water. Indeed, absorption was usually considerably in excess of preceding Cl losses. For the adult, the ratio of Cl uptake to Cl losses varied in seven animals from 1·5 to 21·3, and in eight ammocoetes from 1·0 to 6·5. Some confirmation of the effect on internal Cl levels of washing out in distilled water, followed by a period in tap water, was obtained by determinations made on the serum of ammocoetes, both at the end of the distilled water period and after 14 days in tap water. In this experiment twenty-two animals were used, six being used as controls on which Cl determinations were made at the outset. The difference between the control group and the distilled water group, although not large appears to be significant (Table 5). Values obtained for animals after uptake in tap water are significantly higher than those of the control group, although some increase in Cl levels was to be expected in starving animals, even where they have not been previously washed out in distilled water. This marked excess of Cl uptake over previous Cl losses might be explained by supposing that, in the early stages of starvation, osmotically active materials are metabolized and the total concentration maintained by an increase in the Cl fraction. If this were so, then the uptake of Cl after washing out in distilled water would have two aspects: the replacement of the Cl ions actually lost to the distilled water and in addition the delayed substitution of Cl for other metabolites.

Table 5. *The effects on the serum* Cl *concentration of ammocoetes of a period of* 14 *days in distilled water followed by* 14 *days in tap water*

	Cl concentration in mM. Cl/l.		% increase (+) or decrease (−) on control group
	Mean	S.E.M.	
A. Control animals	61·0	±1·85	—
B. After 14 days in distilled water	54·1	±1·27	−10
C. After 14 days in tap water	71·0	±1·6	+16

For difference in means: A and B $t=3{\cdot}08$, $P=0{\cdot}01$.
A and C $t=3{\cdot}84$, $P<0{\cdot}01$.

IV. THE PERMEABILITY OF THE INTEGUMENT TO WATER

In both species the osmotic uptake in tap water has been measured by weighing animals with the urino-genital papilla ligated. Under these conditions *planeri* adults survive for at least 4 hr., and the maximum weight increase for such a period was 22%. For at least the first 2 hr. the increase in weight appeared to be linear and the rates of uptake quoted are for this period. *Fluviatilis* survived for more

than 30 hr. with weight increases of up to 30%, and over the first 24 hr. no decrease in the rate of uptake could be detected. For this species rates cited are those measured over the first period of 3 hr.

For nine adult *planeri* weighing from 2·3 to 4·7 g. the percentage weight increase per hour varied from 3·8 to 7·6 at 12–13° C., with a mean value of 5·6. At the same temperature six adult *fluviatilis* (21–54 g.) in October and November showed weight increases from 1·0 to 1·4%/hr. with a mean value of 1·25, while two further animals tested in April gave increases in weight of 1·7 and 1·9%. From these figures the mean rates of urine flow in fresh water may be expressed as 1·3 ml./g./day for *planeri* and 0·3 ml./g./day for *fluviatilis*. The urine output in *fluviatilis* was measured directly by Wikgren (1953) at various temperatures by keeping the animals with only the anterior region of the body immersed in water. Interpolating from Wikgren's data a value of 0·3 ml./g./day is obtained for a temperature of 13° C., which is identical with that found by weighing. Such agreement provides support for the view that the branchial epithelium plays the major role in water transport.

In considering the different rates of uptake of the two species, account must be taken both of the disparity in size and the rather higher osmolar concentration of the serum in *fluviatilis*. Taking the mean length of the *fluviatilis* used in these experiments as about 2·4 times that of *planeri*, the surface area of the larger form (assuming the proportions of the two species to be similar) may be expressed as $2{\cdot}4^2\,A$, where A is the surface area of *planeri*. Similarly, if W is the weight of *planeri*, the weight of *fluviatilis* may be represented as $2{\cdot}4^3W$. From the observed mean weight increases, the weight of water entering the body of the animal per hour may be given as $\frac{5{\cdot}6\,W}{100}$ for *planeri* and $\frac{1{\cdot}25\times 2{\cdot}4^3\,W}{100}$ for *fluviatilis*. If permeability is expressed as grams of water passing through unit surface per hour for a concentration gradient of I mole, then the permeability of the two forms will be given by

$$\frac{5{\cdot}6\,W}{100\mathrm{A}\times 0{\cdot}11}=\frac{0{\cdot}51\,W}{A} \quad \text{for } \textit{planeri} \text{ and}$$

$$\frac{1{\cdot}2\times 2{\cdot}4^3\,W}{100\times 2{\cdot}4^2A\times 0{\cdot}14}=\frac{0{\cdot}21\,W}{A} \quad \text{for } \textit{fluviatilis}.$$

Thus the permeability of the integument to water would appear to be about twice as great in *planeri* as in *fluviatilis*.

V. THE EFFECTS OF SALINE SOLUTIONS

Observations have been made on the osmotic loss of weight in sea water and various dilutions of sea water with tap water. Experience showed that, even in solutions which were well below the level of concentration of the body fluids, there was in the case of adults and ammocoetes of *planeri* an initial loss of weight over and above the normal rate of metabolic loss for animals kept continuously in tap water. This excessive loss of weight was, however, compensated when animals were returned from the saline solution to tap water. Comparison of the behaviour of ligated and

normal animals in saline solutions suggests that these initial losses are due to a delay in the reduction of the urine flow from its high fresh-water rate.

Although both ammocoetes and adult *planeri* survive well in diluted sea water, Ringer or even pure NaCl solutions of less than about 120 mM., they rapidly succumb in higher concentrations. In this respect the adult appears to be less resistant than the ammocoete. In sea water diluted to about 270 mM. the adult survived for about 6 hr., but pure sea water was generally lethal in 2–3 hr. By contrast, *fluviatilis* in October and November survived direct transfer to pure sea water for at least 6 hr. and in some instances tolerated indefinitely 270 mM. sea water. In both species a loss of weight of 20–25% of the initial body weight appeared to be critical from the point of view of survival. Recovery, is, however, usually possible if the animal is returned to fresh water and the weight lost is rapidly regained. Indeed, there is a marked tendency for such animals temporarily to overshoot their initial weight.

In experiments with *planeri*, weighings were carried out in pure sea water and sea water diluted to approximately 360, 270 and 180 mM. With the exception of the second of these solutions, the same range was tested on *fluviatilis* in October and November. In the case of *fluviatilis*, weighings were made at two-hourly intervals up to 6 hr. or longer and, especially in the higher concentrations, there was a marked reduction in the rate of loss after the first 2 hr. Observations on *planeri* were made at hourly intervals and, in those solutions in which the animal survives for several hours, the rate of loss remained approximately linear. In Fig. 2 the osmotic loss/hr. expressed as a percentage of the initial weight is, in the case of *planeri*, that recorded after the first hour, and in the case of *fluviatilis* that recorded after the first 2 hr. in the solution. The osmotic gradients have been taken as the differences between the mean osmolar serum concentration (Table 2) and the estimated total concentration of the sea water.

Values obtained for the ammocoete and adult *planeri* seem to show a wider scatter than could be accounted for, either by weighing errors or individual differences in osmolar concentration, and losses recorded in early April were distinctly lower than those obtained the previous year in late April and May. Ammocoete values, while tending to be higher than those for the adult, show such a wide range that the differences are of doubtful significance. Examination of the observations in Fig. 2 suggests several points of interest:

(1) The curves produced do not pass through the origin. There is little doubt that this may be attributed to the initial delay in the adjustment of urine flow.

(2) For *planeri* the osmotic losses are lower than would be expected from the observed rate of osmotic uptake in fresh water. This can hardly be accounted for by the effects of the water losses on the internal concentration, since the same conditions apply (although in the reverse sense) in the determinations of osmotic uptake. It would be explained if it is assumed that the integument is readily permeable to the inward diffusion of ions.

(3) For *fluviatilis* osmotic losses tend to be rather greater than expected. As this is especially true for the first period of 2 hr. it is thought to be due to the loss of mucus.

(4) As a consequence of (2) and (3), while the rate of uptake in fresh water is over four times as great in *planeri* as in *fluviatilis*, the osmotic losses are in general no more than twice those observed in the larger species. If the interpretations which have been advanced are valid, the relative rates of uptake of the two forms have more significance than the rates of osmotic loss in relation to the physiological effects of direct transfer from fresh to salt water.

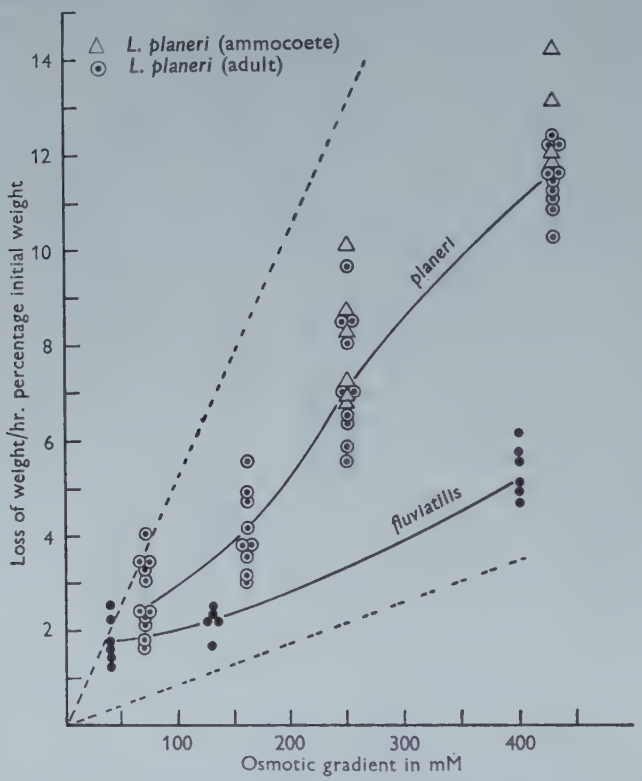

Fig. 2. Osmotic losses of water in various concentrations of sea water. The dotted lines indicate the rate of loss expected from the observed rates of osmotic uptake.

The swallowing of sea water, which is such an important factor in the regulatory mechanisms of marine teleosts, does not appear possible in the adult *planeri* because of the occlusion of the foregut which takes place at metamorphosis (Weissenberg, 1926; Keibel, 1927), but the possibility remains that it might occur in *fluviatilis* when transferred from fresh water to sea water, as in this form closure of the gut does not appear to be complete until the animals become sexually mature in early spring. However, in view of the fact that, during the limited period of the present observations on these animals, osmotic losses in sea water were greater rather than less than expected, such a possibility would seem to be excluded.

Attempts were made, during late autumn and winter, to acclimatize *fluviatilis* to gradually increasing concentrations of sea water, but these failed to produce any

evidence of regulatory processes. Starting from an initial concentration of about 80 mM. sea water, vapour-pressure and Cl determinations were made on the serum of pairs of animals killed at intervals as the concentration of the water was increased. The animals showed no obvious signs of abnormal behaviour until the water concentration exceeded 250 mM. and none died until the end of January when the concentration had reached 300 mM. Yet neither the values for Cl concentration nor the values for osmolar concentration gave any hint of regulation, and the animals became completely isosmotic beyond external concentrations of 200 mM. The last survivor died in February when the water concentration had reached 340 mM., after 6 weeks in water of 200 mM. and upwards (Fig. 3). As it is likely that the ability of these animals to regulate the internal concentration is lost soon after they enter the estuaries in the autumn and early winter, these attempts were presumably made too late in the season and were too prolonged.

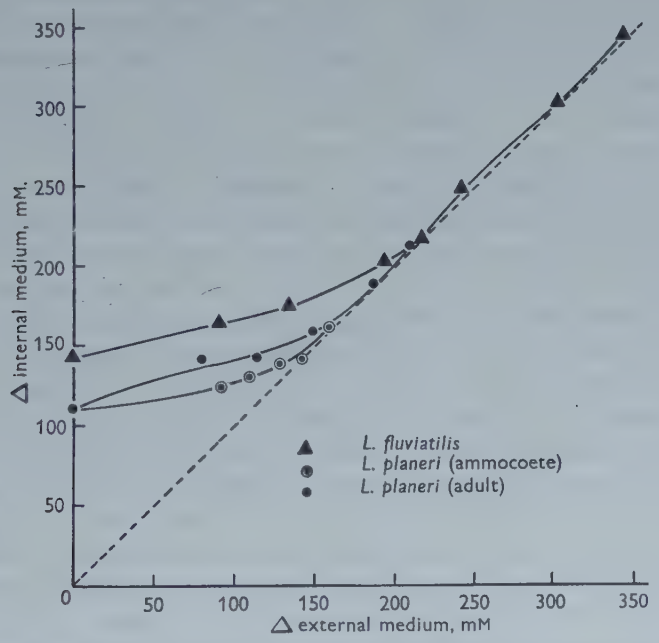

Fig. 3. Acclimatization of ammocoetes and adults to increasing concentrations of sea water.

Similar tests have been carried out on ammocoetes and adults of *planeri* and, except for the greater tolerance shown by the ammocoete to a raised internal concentration, the general trend was similar in both. Up to about 140 mM. the internal concentration remained above that of the water, but beyond this the animals approached a condition in which they were isosmotic and the concentration of the body fluids followed passively the increases in concentration of the external medium. The adult *planeri* began to succumb once the water reached 140 mM. and none survived beyond 160 mM. A few ammocoetes, on the other hand, survived in water of 200 mM., although they were by this time in an abnormal state.

VI. DISCUSSION

Apart from the total concentration of the blood, which is lower in *planeri* than in the anadromous lampreys, it appears that the differences in fat, water and Cl content between the ammocoete and adult *planeri* and the adult *fluviatilis* are to be attributed to the period of starvation which, in both species, precedes sexual maturity. In both ammocoete and adult the effect of fasting is to increase the water content of the body and raise the Cl levels of the body fluids and tissues. The wide variations in the total Cl content of the ammocoete throughout the year and the changes in the Cl fraction of the total concentration shown by both *planeri* and *fluviatilis* emphasize the importance of the part played by Cl metabolism in the osmotic regulation of these animals in fresh water.

The increasing replacement of organic by inorganic constituents in the body fluids which accompanies sexual maturity is made possible by the ability to absorb ions from extremely low dilutions. Since the Cl ion is taken up in face of an increasing concentration of this ion in the body, it would not appear that the absorptive mechanisms are activated specifically by Cl depletion; a point which is further emphasized by the marked excess of Cl uptake over previous Cl losses which has been observed in animals transferred to tap water after a period of depletion in distilled water. The various observations which have been made on Cl metabolism are consistent with the view that it is changes in total concentration rather than Cl (or Na) ions that activates the absorptive mechanisms.

In *planeri* at least, the permeability of the skin and branchial surfaces to water is considerably greater than that recorded for teleosts in fresh water, even when allowances are made for differences in size and concentration gradients. For *Salmo irideus* and *Carassius auratus* the figures given by Krogh (1939) range between 0·04 and 0·1 ml./g./day, for *Cyprinus* and *Anguilla* (Smith, 1929) 0·06–0·15 ml., and for *Ameirus* (Marshall, 1934) 0·3 ml. Moreover, this high rate of filtration in the kidney of the lamprey is achieved, not by a large number of discrete glomeruli, but by a small number of giant rope-like structures, formed by the fusion of separate glomeruli in the posterior part of the ammocoete kidney (Wheeler, 1899). The formation of large volumes of urine by the comparatively small filtration surface of such a kidney suggests a high hydrostatic pressure in the glomerular capillaries and lends added significance to the presence of valves at the origin of the segmental arteries (Young, 1950). Although no direct determinations have been made, the information obtained on urine flow and Cl losses leaves little doubt that the concentration of Cl in the urine must be very low, and that the kidney tubule is able almost completely to reabsorb Cl ions. Thus, taking a daily output of 3 μM. Cl/g. with a urine flow of 1·3 ml./g. and assuming all the Cl to be lost in the urine, the concentration of this ion could not be more than 2·3 mM., which is less than that observed by Krogh (1939) in the urine of *Salmo irideus* and *Carassius auratus*.

Whatever may be the explanation for the reduced rate of osmotic uptake and loss of water through the integument of *fluviatilis*, there can be no doubt of its

physiological significance for an animal which, in the course of its life history, changes from one medium to another. Fontaine (1954) has drawn attention to the relation between size and the amplitude of migration in those groups such as the Petromyzontidae and Salmonidae, which include both migratory and non-migratory forms, and indeed it may well be that increased size is in itself an important factor in the ability of the animal to cope with changes in salinity, through its effects on the relative surface area of the integument.

VII. SUMMARY

1. The mean osmolar concentration (expressed as mM. NaCl/l.) of the serum for *Lampetra planeri* was found to be 110 mM. in the ammocoete and 113 mM. in the adult. For adult *L. fluviatilis* serum in November the mean osmolar concentration was 143 mM. and in March 136 mM.

2. In adult *planeri* the Cl concentration of the body fluids accounted for nearly 90% of the total concentration as compared with only 58% in the serum of the ammocoete. Adult *fluviatilis* kept in tap water throughout the winter appeared to show an increase in Cl levels.

3. Evidence has been produced to support the view that the differences observed in water, fat and Cl content of ammocoete and adult *planeri* and adult *fluviatilis* are consequences of the period of starvation preceding sexual maturity.

4. Pronounced seasonal variations have been found in the fat and Cl content of ammocoetes of *planeri*.

5. The osmotic uptake of water has been determined for adults of both species in fresh water. In *planeri* the rate of uptake for unit body weight is about four times as great as in *fluviatilis*.

6. Observations have also been made on the osmotic loss of water of ammocoete and adult *planeri* and of adult *fluviatilis* in sea water of different concentrations. For unit body weight osmotic loss of water in *planeri* is about twice that of *fluviatilis*.

7. Total losses of Cl have been determined for ammocoete and adult *planeri* in distilled water and their effects on internal Cl levels have been studied. Observations have also been made on the active uptake of Cl.

8. Acclimatization experiments on ammocoetes and adult *planeri* and on adult *fluviatilis* have failed to produce any evidence of regulation in sea water.

I should like to take the opportunity of thanking Prof. J. Z. Young, F.R.S., for his help and guidance on many occasions during the preparation of this paper and also Dr J. D. Robertson, who was good enough to read and criticize the manuscript. I am also indebted to many of my colleagues for their help in the course of my work and to others who have assisted me in the collection of the animals, particularly my wife and Mr J. R. O'Connor. Acknowledgements are also due to Dr J. D. Jefferson for his helpful suggestions in connexion with permeability and to the Governors of the Bristol College of Technology for the provision of facilities for this research.

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ADDENDUM

Since the preparation of this paper some information has been published by Morris (1956) on the osmo-regulatory ability of fresh-run and mature river lampreys subjected to gradually increasing concentrations of artificial sea water. These experiments confirm my own experience that there are marked differences in the water permeability of the integument and in tolerance to sea water of fresh-run and mature animals. In some fresh-run animals Morris found definite evidence of Cl regulation in diluted sea water (less than 270mM.) although the osmotic concentration of the plasma reached levels above those of the water. Urine flow was measured directly by Morris in two ways; by cannulation after anaesthetization with chlorbutol and by a divided chamber method similar to that employed by Wikgren (1953). Using the latter method the rate of urine flow in fresh water was found to be 341·9 ml./Kg./day at 16–18° C., a figure which agrees very

closely with those obtained by both Wikgren and myself (when corrections are made for temperature). The cannulation method, however, gave a much lower figure (155·8 ml./Kg./day), which Morris believes to be the more reliable, but I find it difficult to accept his suggestion that the higher figures may be due in part to shock diuresis or to damage inflicted on the skin by the membranes used in divided chamber experiments. The fact that, in my own observations, the rate of weight increase in ligated animals has remained approximately linear for periods up to 30 hrs. and the close agreement reached by three separate observers using different handling techniques, seem to discount the influence of shock, while the effect of local skin damage would hardly be very serious, if as seems likely, the branchial epithelium is the main source of water uptake. Furthermore, in the absence of controlled experiments, it would not be safe to exclude the possibility of antidiuretic effects through the use of chlorbutol as an anaesthetic.

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CONTROL OF THE MELANOPHORES OF THE MINNOW (*PHOXINUS PHOXINUS* (L.))

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(With Plates 6 and 7)

I. INTRODUCTION

The early work of von Frisch (1911) showed that in the minnow (*Phoxinus phoxinus* (L.)) the melanophore motor connexions with the brain pass through the spinal cord as far as vertebra 15 and there emerge to pass into the sympathetic chain to innervate the skin melanophores through spinal and cranial nerves (Text-fig. 1 *a*). Section at any of these levels always resulted in a dispersion of the disconnected melanophores; on the other hand, electrical stimulation caused an aggregation of the affected melanophores. Von Frisch concluded that the melanophores were innervated by a single system of aggregating fibres and that when these tonic influences were removed the melanophores reverted to a resting state of complete dispersion.

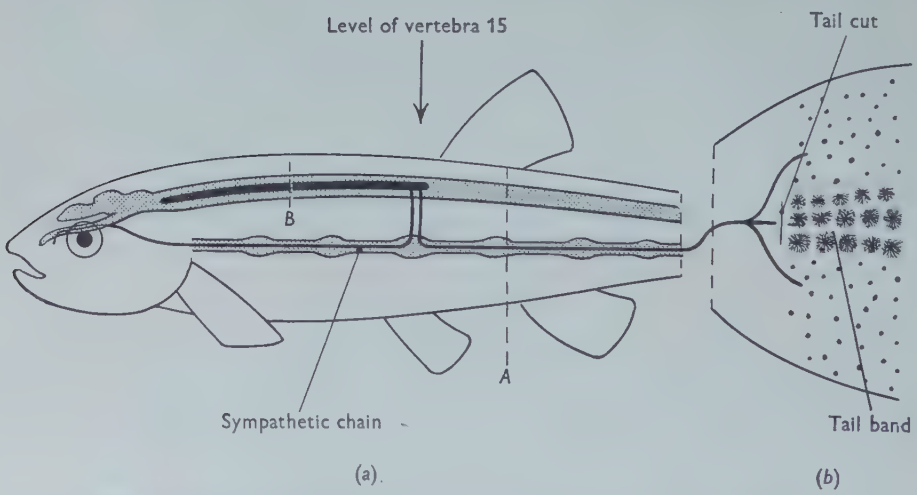

Fig. 1. (*a*) Diagram of the distribution of the chromatic nerve fibres of the minnow (after von Frisch). (*b*) Diagram showing cut made in the base of the caudal fin, resulting in a band of dispersed melanophores in a white-adapted fish.

Parker (1948) and his school, from experiments on section of nerve fibres supplying bands of caudal melanophores in *Fundulus* and *Ameiurus*, maintained that the innervation of teleostean melanophores is double. The initial dispersion in the white-adapted fish, which persists for some time after nerve section, is held to be

the result of stimulation, by cutting, of the dispersing-fibre component. It was shown, for example, that a second dispersion resulted from section of nerve fibres already disconnected from central nervous influences by a previous cut. Subsequent to the initial dispersion, the melanophores of the affected band were observed to regain the capacity to react to background change. The conclusion was drawn that the still intact aggregating- and dispersing- fibre endings innervating the melanophores surrounding the band liberate neurohumours that diffuse into the margins of the band. Parker (1940) was aware of the possible participation of pituitary colour-change hormones, but both he and Matthews (1933) obtained similar results on hypophysectomized fish, which indicated that the initial dispersion and subsequent reactions were not significantly dependent on the activities of pituitary hormones.

Waring (1942), Young (1950) and others have called attention to the difficulties in Parker's hypothesis, and they considered his evidence for double innervation in teleosts inconclusive. They favoured a single system of aggregating fibres antagonized by a blood-borne pituitary dispersing hormone.

There is thus a confusion of opinion over the effects of nerve section on the melanophores and on the related question of double or single innervation. In the minnow, the subject of this present work, Healey (1940, 1951) has given evidence for both pituitary dispersing and aggregating hormones, supplementing the nervous control of the melanophores described by von Frisch. These endocrine factors have therefore to be taken into account in considering the reactions of band melanophores in the fish. No experiments on caudal bands in minnows have yet been reported. Using the technique described below, answers to the following questions are sought. Does nerve section result in a dispersion of the related melanophores? If so, can the dispersion be attributed to pituitary hormones or to stimulation of dispersing fibres or is there some other mechanism involved? Do the subsequent activities of these melanophores indicate the presence of aggregating and dispersing neurohumours? Do the results favour single or double innervation of the melanophores?

II. METHODS

The minnows used were collected from Lake Blaenmelindwr in Cardiganshire and varied from 5 to 7 cm. in length. The colour-change reactions of each fish were tested macroscopically before it was used for an experiment.

(*a*) *Method of tail chromatic nerve section*

In the past the teleosts used for caudal band experiments (cf. Parker, 1948) were suitable for this purpose because the inter-radial tissues of their caudal fins are well supplied with melanophores. Unfortunately melanophores are almost absent from the inter-radials of the minnow (Text-fig. 2). However, the integument of the muscular region *A* is well supplied with melanophores. It receives its vascular supply from the blood vessel arc *B*. By making a vertical incision at *C* the chromatic fibres supplying a band of melanophores in region *A* could be severed without

interfering with the blood supply to this region. The cut was made with a new piece of safety-razor blade with a cutting edge 0·5 mm. long, held in an artery forceps and used chisel-fashion. Viewed under a binocular microscope, the blade was first inserted between the scales before pressing down. This method avoided direct pressure on the melanophores, which would result in their rupture. The resulting area of melanophores in *A* with sectioned nerves will be referred to as a 'tail band', since the term 'caudal band' of other workers refers more exactly to a band in the caudal fin tissues.

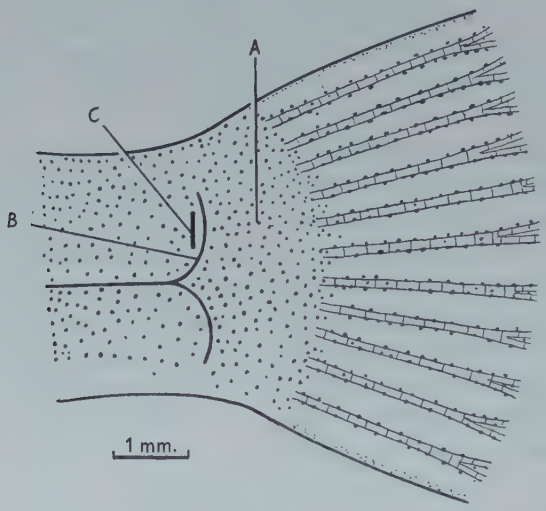

Fig. 2. Diagram of the tail and base of the caudal fin of the minnow. The melanophores are aggregated. *B*, the blood vessel arc. *C*, position of cut to sever the motor fibres supplying melanophores in region *A*. Further explanation in text.

(*b*) *Apparatus for observation of melanophores*

Observation of the tail band by placing the fish on a microscope stage is out of the question, since handling can result in rapid changes of the melanophores. A method of continuous observation without disturbing the fish was found essential, but confinement in a tube for this purpose resulted in abnormal chromatic responses (Gray, 1956). Healey (1956) designed a method of continuous observation to overcome this difficulty, the tail region only being confined in an observation cell. The fish was previously subjected to spinal section posterior to vertebra 15 (see Text-fig. 1*a*). In this way afferent stimuli from tail to brain are eliminated without interfering with chromatic control, so that the fish can be anchored by securing a thread to the tail region. Spinal section also greatly reduces the locomotive powers of the fish. Before being introduced into the apparatus it was trained to remain stationary by confining it in a tube for several days. Healey's method has been adopted here, although the continuous observation apparatus described below contains certain modifications.

The observation cell (3, Text-fig. 3*a*) was constructed out of transparent Perspex. Tube (2) conducted water circulating between cell and tank. Tube (4) housed a cotton thread (5), sewn through the base of the anal fin to anchor the fish in position. When the tail cut was made (II*a*) the thread was paid out or pulled in through tube (4) with the right

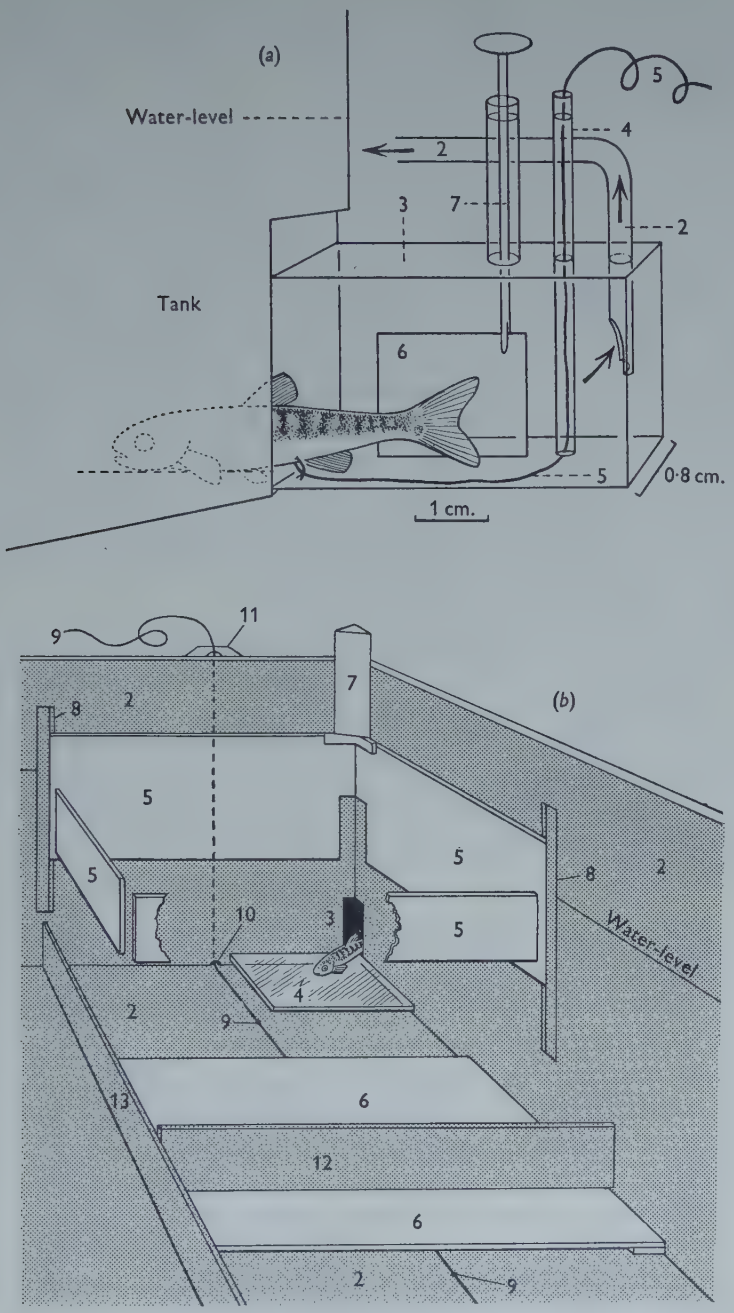

Fig. 3. The continuous observation apparatus (modification of Healey's method). (*a*) The observation cell, projecting from the corner of the tank. (*b*) Anterior view of the tank, looking into the corner. For explanation see text.

hand, the fish being removed and returned to the apparatus with the left hand. The transparent panel (6) could be moved by a swivelling rod (7) so that the tail of the fish was held lightly in position against the near wall of the cell during a photomicrographic exposure. In this way the surface of the tail region was held at right angles to the microscope objective.

The tank (Text-fig. 3*b*, 2) was constructed from black Perspex. An aperture (3) led into the observation cell, projecting from the corner of the tank. The fish rested on the raised transparent platform (4) so that the white Perspex panel systems (5 and 6) could slide around and below it respectively. (5) was operated by vertical movements of arm (7), and (6) was moved horizontally by pulling on the nylon thread (9) from outside the tank. (9) passed out of the tank by hole (10) and watertight sleeve (11). A similar arrangement at the other end of the tank allowed (6) to be pulled in the other direction. (8) and (13) are guide rails for (5) and (6). Vertical black panels (12 and 13) obscured water-circulating and temperature-control apparatus in the other part of the tank (see Healey, 1956) from the visual field of the fish. (12) was mounted so that (6) could slide under and be obscured by it. Thus when the white panel systems (5 and 6) surrounded the fish a continuous white background was presented. Withdrawal of these panels gave black background presentation. In this way the background could be reversed from outside the tank without disturbing the fish. Tank water was controlled at 16° C. and a 15 W. clear bulb suspended 46 cm. above the fish gave constant illumination. Visual and photomicrographic recordings were made with a microscope mounted horizontally on a bench capable of easy vertical and horizontal adjustment of position (Healey, 1956).

This apparatus proved suitable for observations on fish subjected to white backgrounds and background reversals. However, melanophores were never observed to remain dispersed during prolonged black background presentation, a reaction attributed to the effects of confinement (cf. Gray, 1956). For this reason experiments involving prolonged black-background presentation were avoided.

Full records of experiments and series of photomicrographs can be referred to in a Ph.D. thesis by the author (Department of Zoology, University College of Wales, Aberystwyth, 1955).

III. THE EFFECTS OF MELANOPHORE NERVE SECTION IN THE WHITE-ADAPTED MINNOW AND SUBSEQUENT REACTIONS OF THE BAND MELANOPHORES TO BACKGROUND REVERSALS

A fish was subjected to spinal section (method of Healey, 1940) posterior to vertebra 15, trained by tube confinement (see Healey, 1956) over a black background for 5 days and introduced into the continuous observation apparatus (II*b*). The fish was subjected to a white background for 16 hr. and became pale (Pl. 6*a*). Then the tail cut was made (II*a*) and the result was a dispersion of a band of melanophores posterior to the cut (see Text-fig. 1*b*), the dispersion being complete in 15 min. (Pl. 6*b*). In the following 12 hr. the band melanophores slowly returned to the aggregated state, those at the margins of the band aggregating ahead of the more internal ones (Pl. 6*c–e*). 24 hr. after the tail had been cut the background was reversed to black. As a result both the normal and band melanophores dispersed, the latter appearing to disperse at a slightly slower speed, but uniformly throughout the band and *not first at the margins*. Both normal and band melanophores reached

a more or less uniform state of dispersion in 30 min. (Pl. 6*f*). After a further 30 min. the black background was replaced by white, and both normal and band melanophores aggregated at similar speeds, without any differences between the margins and centre of the band. Similar results were obtained in experiments on five other fish.

IV. THE EFFECT OF PITUITARY HORMONES ON TAIL BANDS

(*a*) *Nerve section in fish after hypophysectomy*

Experiment III was repeated on eight fish hypophysectomized 6 days previously (Healey's method of operation, 1940). A tail cut resulted in band formation as in III, the dispersion taking 15 min. Band fading also followed the same course in these white-adapted fish, the aggregation being complete in 12 hr. Again this initial fading was marginal, the outer band melanophores aggregating before the more central ones. As in III, a black background was afterwards substituted for the white one. The result was first a dispersion of the normal melanophores, followed later by a dispersion of the band melanophores, and this time, unlike the reaction in the normal fish, the more marginal band melanophores dispersed ahead of the more central ones (Pl. 7*a–d*). One hour after black-background presentation, white was resubstituted. The result was an aggregation of the normal melanophores, followed by an aggregation of the band melanophores. Again the effect began at the margins.

After the experiments the heads of the fishes were sectioned. In all cases a large portion of the pituitary gland had been removed, although small fragments of the more inaccessible anterior region remained. The results therefore indicate that pituitary hormones are not necessary for the initial dispersion of the band melanophores following section of their nerves, nor for their subsequent re-aggregation in the white-adapted fish. The band melanophores reacted to subsequent background reversals in these hypophysectomized fish, but in this case their reactions differed from those of normal fish in that the dispersion and aggregation began at the band margins and proceeded inwards.

(*b*) *Does the initial dispersion still take place when the pituitary dispersing hormones are eliminated by prolonged white-adaptation?*

The results of IV (*a*) indicated that blood-borne pituitary hormones were not necessary for the initial dispersion resulting from nerve section. This matter can be tested in another way. Healey (1951) showed that from 60 to 80 hr. of exposure of a previously fully black-adapted fish to a white background is necessary to reduce dispersing pituitary hormone effects to a minimum and increase aggregating pituitary hormonal effects to a maximum. Accordingly, six fish were maintained on an illuminated white background for the much longer period of 12 days. After this time tail cuts were made and microscopic examination 15 min. later showed that, as a result, all six fish had developed tail bands with fully dispersed melanophores.

V. THE EFFECTS ON BAND MELANOPHORES OF ELECTRICAL STIMULATION OF FIBRES INNERVATING NEARBY MELANOPHORES

Electrical stimulation of chromatic nerves causes an aggregation of the melanophores they innervate (p. 448). If Parker (p. 448) is correct it should be possible to detect the activities of aggregating neurohumours liberated in this way from intact nerve endings and diffusing to affect previously dispersed neighbouring band melanophores.

The band melanophores can be dispersed under two different circumstances (see III): (*a*) in the white-adapted fish as an immediate result of nerve section (the initial dispersion), or (*b*) as a result of subsequent black-background presentation. The effects of electrical stimulation of the intact nerves were therefore observed under both these conditions.

For electrical stimulation bipolar electrodes were used, coupled in series with a 500,000 ohms variable resistance and the secondary of a bell transformer giving 8 V. a.c. output. The primary input was 230 V. 50 c/s. The variable resistance was adjusted to the minimal current needed to cause aggregation of the tail melanophores when the chromatic fibres of the posterior sympathetic chain were stimulated. Tests showed that no tissue conduction to the tail melanophores took place under these conditions; responses were only seen when the electrodes were touching the chain.

Tail bands were prepared in twelve white-adapted minnows and the fish returned to the white background. 90 min. after the cut a fish was captured, killed by decapitation and the haemal canal exposed in the tail preparation by transecting the trunk about 1 cm. posterior to vertebra 15 (Text-fig. 1*a*, *A*). The preparation was then placed on a microscope stage and kept moist with Ringer. In the following 10 min. the preparation darkened and all the melanophores surrounding the band melanophores dispersed so that the band melanophores were indistinguishable from them. The posterior sympathetic chain in the haemal canal was then stimulated for 20 sec. and the result was a complete aggregation of the melanophores outside the band, but the band melanophores remained fully dispersed and unaffected by electrical stimulation. On cessation of stimulation the melanophores outside the band redispersed. Stimulation was repeated on preparations made at hourly intervals on five of the remaining fish. During this time the bands were narrowing in the process of primary fading (see III). Again those band melanophores which still remained dispersed failed to react to electrical stimulation. Thus band melanophores newly dispersed as a result of nerve section show no reactions to the stimulation for 20 sec. of nearby intact nerve endings.

The remaining six pale fish were then transferred to a black background for 4 days to disperse the band melanophores as well as the normals. When preparations from these fish were stimulated both surrounding and band melanophores aggregated at apparently the same rates and all became completely aggregated in 20 sec. The band melanophores appeared to aggregate uniformly and not first at the margins. On cessation of stimulation the band melanophores remained aggre-

gated, whilst the normals redispersed in 2 min. Repetitions of the stimulus resulted in aggregation of the surrounding melanophores, but the band melanophores, having aggregated once, remained in this state throughout the experiment.

VI. DOES SPINAL SECTION STIMULATE DISPERSING FIBRES AND CAUSE A RELEASE OF DISPERSING NEUROHUMOURS AT THE FIBRE ENDINGS?

In the white-adapted fish, section of the spinal cord anterior to vertebra 15 (see Text-fig. 1) causes a dispersion of the skin melanophores (see Healey, 1940, 1951). If dispersing fibres are involved in this reaction it might be possible to detect a liberation of dispersing neurohumours diffusing into a tail band.

A tail cut was made in a minnow (as in III, see Text-fig. 1*b*), and the fish was replaced in the continuous observation apparatus on a white background. The tail region was photographed to record the position of the resulting band of dispersed melanophores. In the following 12 hr. the band melanophores re-aggregated (as in III), so becoming indistinguishable from the surrounding melanophores. The fish was subjected to spinal section at *B* (Text-fig. 1) 24 hr. after making the tail cut and was returned to the apparatus during recovery from anaesthesia. Observations 10 min. afterwards showed that the band melanophores remained completely aggregated, but the surrounding melanophores (with intact fibres) had dispersed as a result of spinal section. In other words, the spinal fish on a white background had become dark except for the microscopic band.

In the following 48 min. the band melanophores slowly dispersed and the dispersion clearly started at the margins of the band and proceeded inwards (Pl. 7*e*,*f*). The experiment was repeated on five other fish with similar results.

Since the fish was on a white background throughout the experiment the aggregated band melanophores were under the continued influences of aggregating pituitary hormones. In spite of this the band melanophores dispersed marginally, following the dispersion of the surrounding melanophores induced by spinal section. These results can therefore be interpreted in favour of the presence of dispersing fibres. Those still connected to the melanophores surrounding the band appear to be stimulated by spinal section to release dispersing neurohumours, which spread into the band.

VII. THE ASYMMETRIC RESPONSES OF BAND MELANOPHORES

Examination of the melanophores on the margins of a newly dispersed band resulting from a tail cut in the white-adapted fish (III, Pl. 6*b*) showed them to be in a state of asymmetrical dispersion. The processes directed outwards towards the areas of normal melanophores remained much more aggregated than those directed internally towards the symmetrically dispersed band melanophores. This phenomenon has been observed in three other species of teleosts, apart from the minnow (Gray, 1955). It was concluded that only the fibres supplying the inner portions of these individual melanophores had been severed. Previous workers having shown that

a number of fibres can innervate a single melanophore, the incomplete disconnexion of marginal melanophores might be expected.

Whatever the mechanism which operates to cause a dispersion as a result of nerve section, it appears that it need not activate the melanophore as a whole but can be localized to one region of the individual melanophore.

Observations and photomicrographs also showed that in primary fading of the band melanophores (III, Pl. 6*c–e*) not only do the outer band melanophores aggregate ahead of the inner ones, but the outwardly directed processes of the individual melanophores aggregate before the inner processes. In other words the asymmetrical response progresses inwards.

If Parker's interpretation (p. 448) that fading takes place by diffusion into the band of aggregating neurohumours is correct, it would appear that these neurohumours affect not only the melanophores in their entirety, but also localized regions of the individual melanophore.

VIII. REGENERATION OF TAIL BAND CHROMATIC FIBRES

The experiments described in previous sections were all completed within 5 days of making the tail cut. Since workers on other teleosts (see Parker, 1948) have shown that function after regeneration does not commence until 18 days after nerve section, regeneration of the melanophore fibres is not considered a likely explanation of these results.

Histological investigations on the melanophore fibres have so far proved unsuccessful; these fibres appear to be exceptionally refractory to various methylene blue and silver techniques.

However, it was shown (VI) that spinal section results in a dispersion of the normal but not the disconnected band melanophores. The experiment was repeated on a group of twelve minnows, spinal section being carried out at progressively longer intervals after making the tail cut. The differential reaction was observed in six fish up to 14 days after cutting the tail. After 18 days the remaining six fish showed no differential reaction; both normal and band melanophores dispersed simultaneously. Therefore functional regeneration does not appear to take place for at least 14 days.

IX. DISCUSSION

In both the normal (III) and hypophysectomized (IV) white-adapted minnow section of tail fibres results in a distal melanophore dispersion. After a time the band of affected melanophores regains the capacity to react to background colour, not only when pituitary hormones are present but also after hypophysectomy. In both normal and hypophysectomized fish the initial fading is marginal and in the latter the band melanophores react marginally to black and white background presentations. There must therefore be some spread of influence from connected melanophores to those of the band. Observations on the minnow therefore support Parker's observations on other teleosts. These marginal reactions seem to imply the presence of both aggregating and dispersing neurohumours.

Electrical stimulation caused an aggregation of band melanophores with fibres sectioned 5 days previously (V). This would appear to indicate the activities of aggregating neurohumours spreading into the band. It might be expected, however, that the marginal band melanophores would react first, whereas in fact the aggregation appeared uniform (V). Possibly the spread is so swift under these conditions that the aggregation appeared uniform, but further evidence is needed on this point.

The activities of diffusing dispersing neurohumours are indicated when a fish with a faded tail band is subjected to spinal section (VI). It is difficult to explain the marginal dispersion of the tail band melanophores in this case except on the basis of spreading dispersing neurohumours.

The above factors in the minnow tend to confirm Parker's observations on other teleosts. If they are interpreted in favour of two neurohumours then obviously the melanophores are doubly innervated.

However, it is difficult to accept Parker's interpretation that the initial dispersion, which persists for 12 hr. in the minnow (III), and for several days in the much larger caudal bands of Parker's (1948) fish, is caused by continued injury discharges in the dispersing (but not in the aggregating) fibre stumps. Yet the phenomenon needs to be accounted for. It does not appear to result from pituitary hormones (IV) activating the melanophores on the removal of central control (see p. 449). Yet nerve section definitely brings into play a persisting dispersing mechanism in the melanophore, rendering it refractory to aggregating neurohumours (either spreading into the band during white background presentation, III and IV, or by electrical stimulation, V), but only for a certain time. A more likely explanation than Parker's is perhaps that removal of central nervous control by nerve section might result in some inherent dispersing mechanism of the melanophore coming into play. Later the melanophores lose their refractoriness to diffusing neurohumours and may even become hypersensitive. This is a likely possibility since mammalian autonomic effectors become sensitized, not immediately but some time after their nerves have been sectioned (see Cannon & Rosenblueth, 1937). Parker (1942) himself made experiments to show that band melanophores became sensitized some time after nerve sections, but held that in the interval dispersing fibre stumps were continuously discharging so that the melanophores remained dispersed.

X. SUMMARY

1. The effects of sectioning the nerve fibres supplying a tail area of melanophores in the minnow under various experimental conditions were studied by a method of continuous observation employing visual and photomicrographic recordings.

2. In the white-adapted fish, nerve section causes a band of melanophores to disperse. The initial dispersion later subsides, the band melanophores aggregating first at the band margins (primary fading). Subsequently the band melanophores react to background reversals in a similar way to normal ones.

3. Hypophysectomy does not interfere with the initial dispersion or primary fading of band melanophores. Furthermore, these melanophores still react to background reversals, but in this case they react first at the margins of the band.

4. Prolonged white-adaptation, favouring maximal activities of aggregating pituitary hormones, does not prevent the initial dispersion resulting from nerve section.

5. Band melanophores with fibres sectioned 5 days previously react to diffusing aggregating neurohumours liberated by electrical stimulation. Melanophores with fibres newly sectioned remain dispersed, refractory to this aggregating agent.

6. When the chromatic fibres at the level of the spinal cord are sectioned in a white-adapted fish with a faded tail band the normal melanophores disperse and later the band melanophores disperse first at the margins. A possible interpretation is that dispersing neurohumours are liberated by spinal section.

7. Asymmetric responses of melanophores indicate that neurohumours can activate localized regions of the individual melanophore.

8. The mechanism of the melanophore dispersion resulting from nerve section is discussed together with the question of single or double innervation and the related activities of aggregating and dispersing neurohumours.

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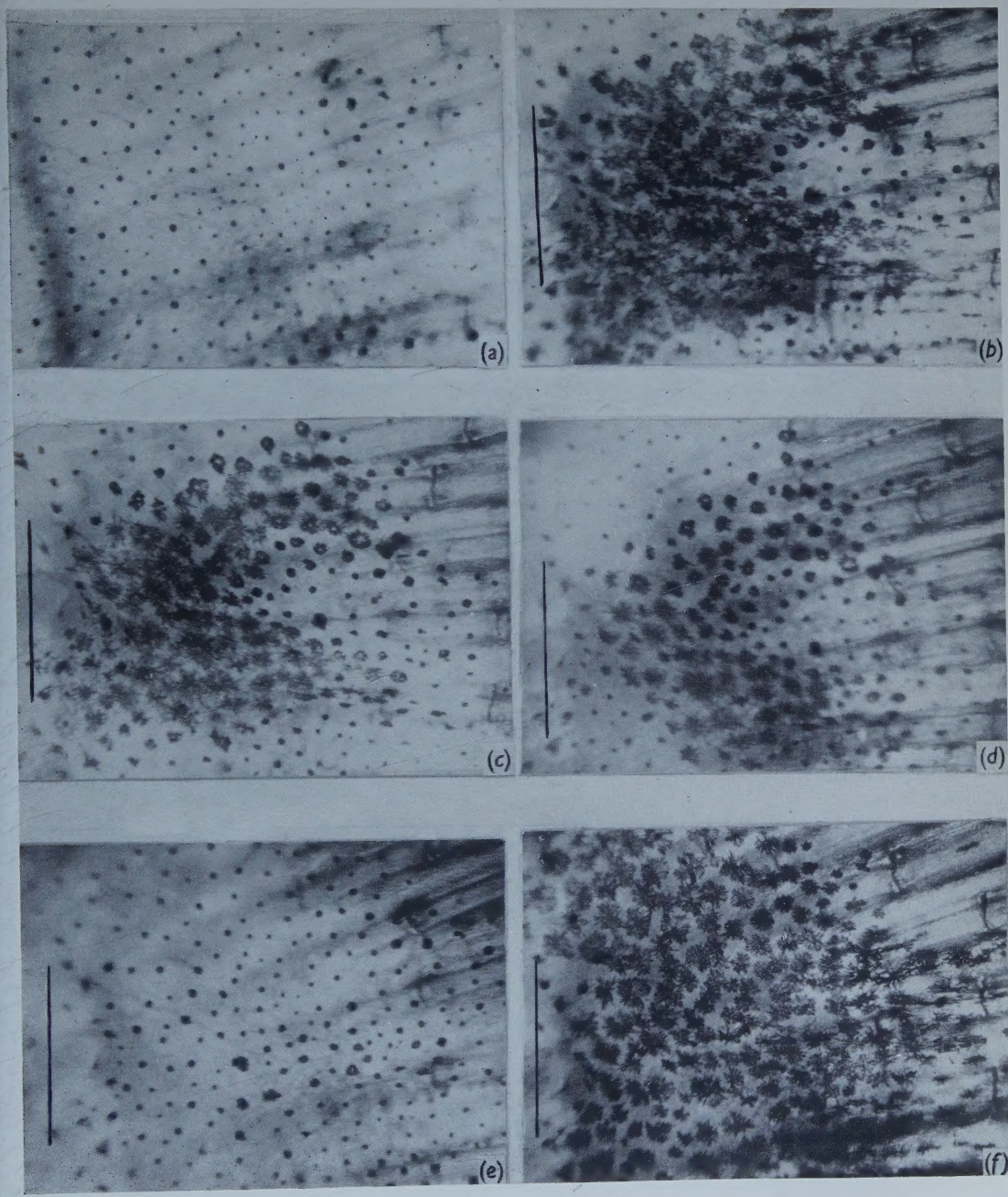
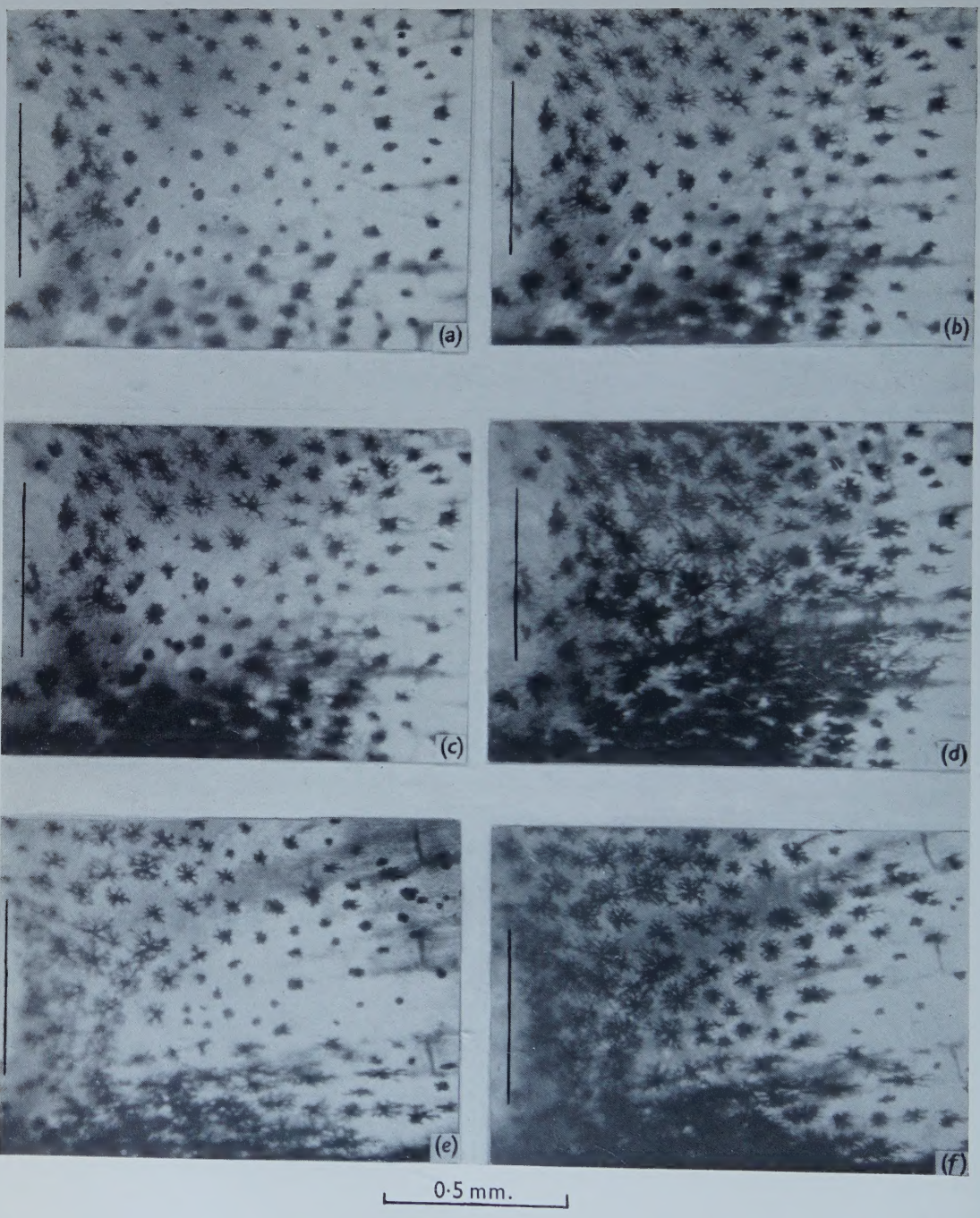


GRAY—CONTROL OF THE MELANOPHORES OF THE MINNOW (*PHOXINUS PHOXINUS* (L.))

(*Facing p.* 458)



GRAY—CONTROL OF THE MELANOPHORES OF THE MINNOW (*PHOXINUS PHOXINUS* (L.))

EXPLANATION OF PLATES

Plate 6

Photomicrographs showing the effects of a tail cut at various stages in the same minnow in the continuous observation apparatus. The line has been added to show the position of the cut. (*a*) Before making the cut. The vertical shadow on the left is of the underlying blood vessel arc (compare Text-fig. 2). (*b*) The fully formed band 15 min. after cutting. (*c*) Marginal fading 2 hr. after cutting. (*d*) Further marginal fading 6 hr. after cutting. (*e*) Almost complete fading 9 hr. after cutting. Note the asymmetric responses of the marginal band melanophores (*b–d*). (*f*) The dispersion of band and normal melanophores 30 min. after black background presentation.

Plate 7

(*a*)–(*d*) Black background presentation after primary band fading in the hypophysectomized fish. The marginal dispersion of the band melanophores subsequent to the dispersion of the surrounding melanophores. (*a*) 2 min., (*b*) 4 min., (*c*) 8 min. and (*d*) 20 min. after black-background presentation. (*e*)–(*f*) Stages in the marginal dispersion of the band melanophores after spinal section in the white-adapted fish.